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Infantile Enteritis Viruses: Morphogenesis and Morphology

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A new virus was found to be associated with acute gastroenteritis in children. In duodenal biopsies, it was observed infecting only intestinal epithelial cells, and it resembled orbiviruses in its morphogenesis. For diagnostic purposes the virus was readily demonstrated by negative staining of fecal extracts. Two forms of particles were seen: double-shelled particles (70 to 75 nm in diameter) resembling those of reovirus but with a sharper outline, and single-shelled particles (60 nm in diameter) with obvious capsomer structure and resembling those of orbiviruses. The morphological resemblance of this human virus to the viruses of "Nebraska" calf scours and epizootic diarrhoea of infant mice is emphasized.

In young children, gastroenteritis or "infantile diarrhoea" is frequently a serious disease requiring hospital treatment to correct dehydration. Despite intensive studies, aetiologic agents such as pathogenic enteric bacteria or protozoa can be incriminated in only a minority of cases (8). We have recently observed a new virus in duodenal mucosa (6) and fecal extracts (7, 10) from a high proportion of young children hospitalized in Melbourne with acute gastroenteritis. The association of this virus with gastroenteritis in children has been confirmed in England (15), Canada (23), and the U.S.A. (17). It has also been demonstrated in limited surveys of fecal samples from children in various other parts of the world (10, 11).

This new virus can not be cultured by routine diagnostic procedures. We report here features of its morphogenesis in biopsy specimens of duodenal mucosa, and of its morphology in negative-contrast electron microscopy. Whereas the virus obviously belongs to the family *Reoviridae*, it cannot be placed in either of the recognized genera (reovirus or orbivirus). We suggest creation of a new genus, duovirus. This new genus could also include morphologically similar viruses involved in the aetiology of gastroenteritis in calves ("Nebraska" calf scours virus) and in mice (epizootic diarrhoea of infant mice, or EDIM virus), and the simian virus SA-11.

MATERIALS AND METHODS

Clinical specimens. All clinical materials were obtained from children admitted to the Royal Children's Hospital, Melbourne. Clinical details and processing of duodenal biopsy samples for electron microscopy have been described (6). Virus-containing extracts were prepared from fecal samples by the method of Bishop et al. (7). Briefly, this involved extraction with trifluor-trichloroethane ("Arklone," Imperial Chemical Industries), precipitation of the virus with 8% polyethylene glycol 6000, and centrifugation of the particles through a layer of 45% (wt/vol) sucrose. The last stage has been modified slightly since the method was published. The deposit obtained after polyethylene glycol precipitation is now dissolved in 4 ml of distilled water. This mixture is layered on to 1 ml of 45% (wt/vol) sucrose in 0.002 M Tris-hydrochloride buffer, pH 7.0, and centrifuged for only 75 min at 100,000 × *g*.

Viruses of calf and mouse origin. A sample of calf scours virus was prepared from a field specimen supplied by T. A. Mason, Department of Veterinary Clinical Science, University of Melbourne, from Werribee, Victoria. Extraction of the virus from the calf feces was carried out as described above for human fecal specimens.

A sample of EDIM virus was obtained from a natural outbreak of diarrhoea in infant mice at the John Curtin School of Medical Research, Canberra, by courtesy of G. M. Woodroofe. Lengths of whole, infected gut, including contents, were homogenized with fluorocarbon, and then particles were centrifuged through 45% sucrose as for human fecal specimens.

Negative-contrast electron microscopy. Virus suspensions were negatively stained with 2% potassium phosphotungstate at pH 7.0 or 6.0, or with 1/10 saturated ammonium molybdate. Specimens were routinely examined and photographed at a magnification of ×20,000 in a Hitachi HU-11A electron microscope. Magnifications were calibrated against catalase crystals (31).

RESULTS

Virus morphogenesis observed in human biopsy specimens. Growth of this virus appears

to be restricted to differentiated intestinal epithelial cells, and it has not yet been adapted to growth in any type of cell culture. Previous light microscope observations suggested that cell damage was most notable in the duodenum (5), and our electron microscope observations on biopsies of duodenal mucosa confirm that evidence of cytopathology associated with virus replication is not difficult to obtain. Even within the very small areas thus surveyed, however, infected cells are scattered among apparently normal neighbors, and no synchrony of infection is evident. Thus the duration of the replicative cycle has not been established, and the sequence of events in morphogenesis can only be suggested by analogy with other members of the reovirus family which have been studied under single-cycle growth conditions (9, 26).

Infected cells are most easily recognized at the stage illustrated in Fig. 1, in which numerous virus particles have accumulated in cytoplasmic vesicles. The microvilli lining the apical surface of infected cells may appear normal or irregular and distorted. The vesicles containing virus particles are in fact distended cisternae of the rough endoplasmic reticulum. Sectioned particles have an electron dense core 33 nm in diameter enclosed by a moderately dense layer interpreted as capsid with an outer diameter of 67 to 70 nm. Some particles lacking the dense core are also evident in Fig. 1. A variable proportion (about 10%) of particles within vesicles were enveloped; their total diameter was then 87 to 90 nm.

Figure 2 illustrates the very characteristic appearance of a mass of convoluted (reticulate) smooth membrane adjacent to enveloped particles at the edge of a vesicle. The budding process by which some particles acquire an envelope when passing into a vesicle is clearly shown in Fig. 3.

Areas of "viroplasm" or "virus factories" similar to those observed in reovirus- or orbivirus-infected cells were encountered less frequently; one is shown in Fig. 4. These are moderately electron dense, granular, or finely fibrillar, and are not membrane bound. Their relatively uniform texture distinguishes them from the surrounding cytoplasmic organelles, but those observed were not very large.

Final dissolution of a necrotic cell is shown in Fig. 5. Virus particles still within membrane-bound vesicles are passing into the lumen

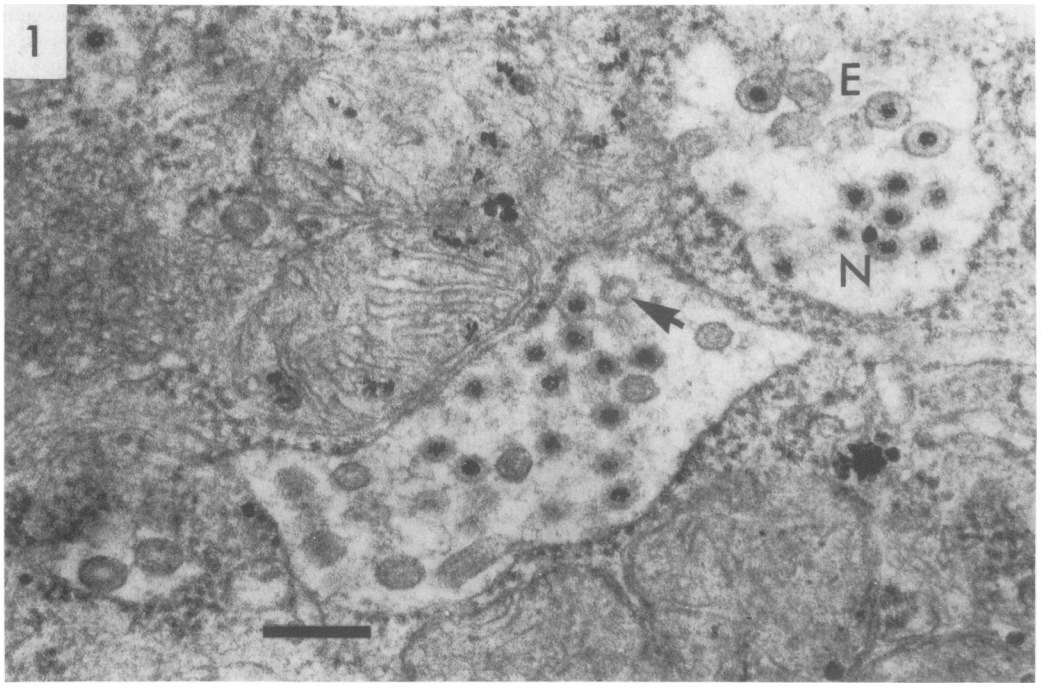

FIG. 1. *Portion of the cytoplasm of a duovirus-infected epithelial cell obtained by duodenal biopsy from a child with acute gastroenteritis, showing duovirus particles within distended cisternae of the endoplasmic reticulum. Both naked (N) and enveloped (E) particles are present. Some particles of each type appear to lack the usual electron-dense core (arrows).* ×*72,000. Bar in this and subsequent micrographs indicates 200 nm.*

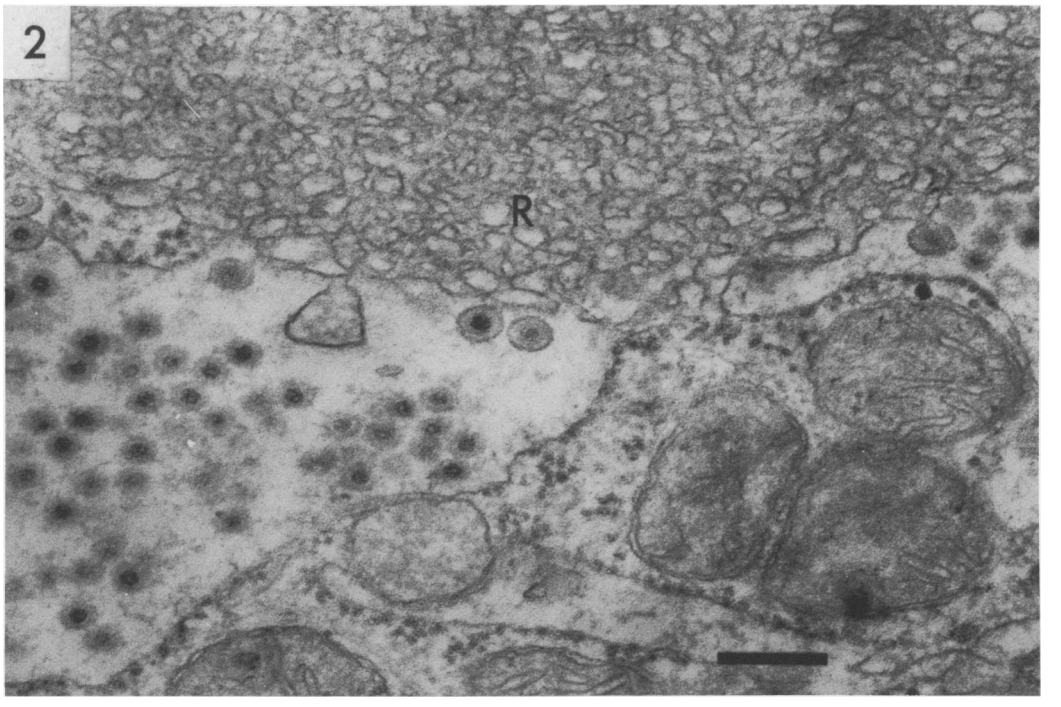

FIG. 2. *Detail of the edge of a cisterna containing virus. Enveloped particles are situated near an intracytoplasmic mass of convoluted smooth membranes (R).* ×72,000.

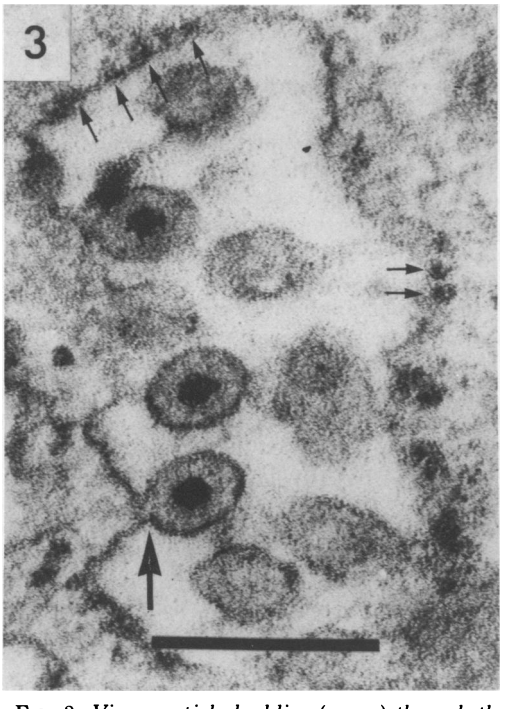

FIG. 3. *Virus particle budding (arrow) through the membrane of a rough endoplasmic reticulum cisterna. Note ribosomes (small arrows) attached to other regions of the same membrane.* ×150,000.

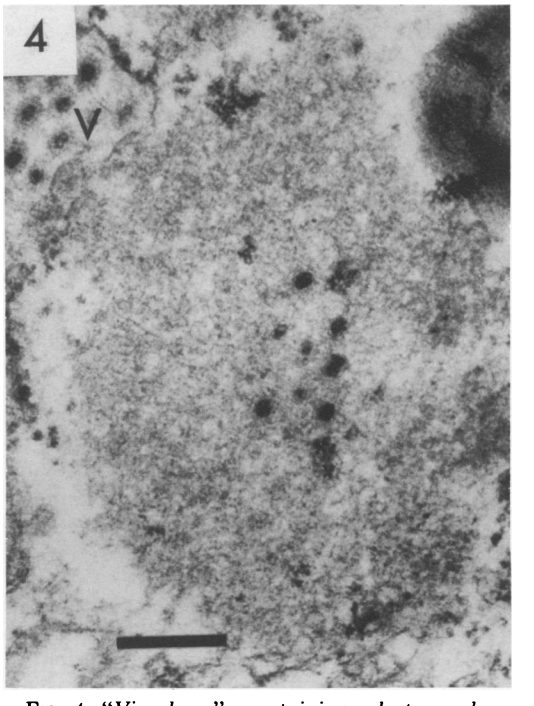

FIG. 4. *"Viroplasm" containing electron dense virus cores in the cytoplasm of a duodenal epithelial cell. An adjacent cisterna contains complete, unenveloped virus particles (V).* ×72,000.

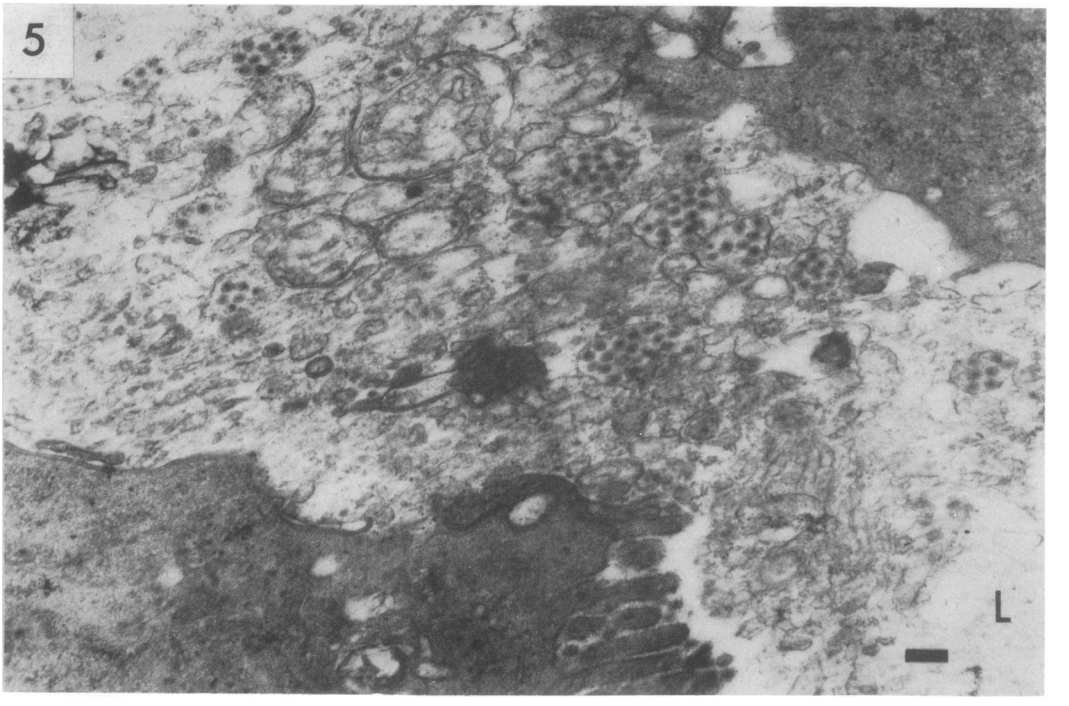

FIG. 5. *Discharge of the contents of a necrotic epithelial cell, including membrane-bound groups of duovirus particles, into the lumen (L) of the small intestine.* ×27,000.

of the gut (lower right), whereas an apparently normal adjacent epithelial cell is partly visible below.

Negative-contrast studies. Virus particle excretion by duovirus-infected children appears to be maximal during day 3 to 4 of their illness; lower concentrations of virus have been detected up to day 10 after onset of symptoms (10). The procedure used for partial purification and concentration of the virus does facilitate diagnostic recognition of the particles by removing a considerable amount of extraneous membranous and particulate material. Nevertheless debris of bacterial origin, assorted bacteriophages, and sometimes adenoviruses are seen, together with variable amounts of unidentified fibrillar material. For diagnostic purposes, we prefer the greater contrast obtained with phosphotungstate, but ammonium molybdate reveals the outer capsid shell of particles more clearly and appears preferable for studies of the fine structure of this type of virus.

Duovirus particles are recognizable by their regularity, size, and surface structure. Specimens may contain predominantly double-shelled or single-shelled particles, or a mixture of both (Fig. 6). Particles with a double-shelled capsid have a diameter of 70 to 75 nm and a characteristically sharp outline. Arrangements resembling large ring-shaped capsomers are sometimes visible (arrow, Fig. 6), but these may be spaced apart by half their width and in other cases overlap. A regular arrangement of hollows surrounded by shared structural units (as proposed by Vasquez and Tournier [28] to explain reovirus surface structure) would appear to us a better description of the general appearance. Portions of disintegrating particles appear to consist of a lattice structure, not separate ring-shaped capsomers (Fig. 7). Very similar fragmentation of reovirus capsids has been reported (2).

Single-shelled particles (60 nm in diameter) exhibit a much more obvious capsomer structure than cores of reovirus particles; they closely resemble typical orbiviruses.

A small proportion of human virus preparations contained flattened tubular structures with a regular surface pattern (Fig. 8). These were shown to be antigenically related to virus particles by immunoelectron microscopy (I. H. Holmes, B. J. Ruck, R. F. Bishop, and G. P. Davidson, manuscript in preparation). The width of the tubules varied (54 to 100 nm, mean 70 nm), but the lattice spacing was constant, about 10 nm. Similar tubules with a 10-nm lattice spacing were also observed in negatively stained preparations of EDIM virus (Fig. 9) and

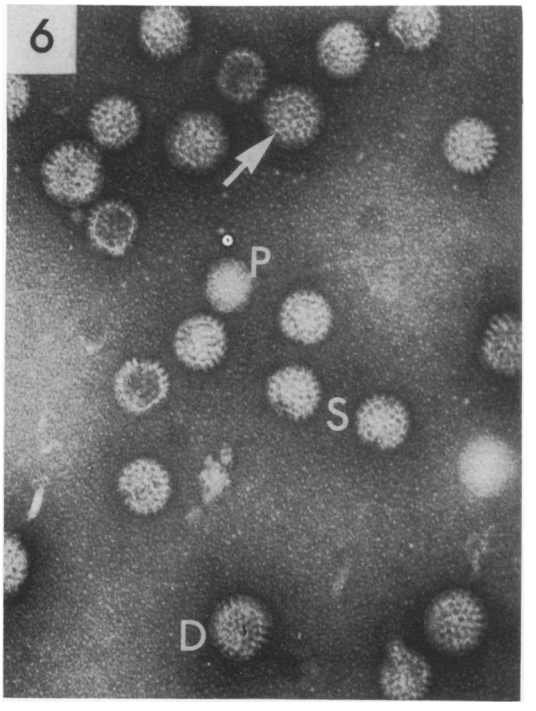

FIG. 6. *Negatively stained human fecal extract containing both double (D) and single-shelled (S) duovirus particles. The surface structure of the outer shell consists of overlapping rather than separate ring-shaped capsomers (arrow). P indicates a bacteriophage particle. Ammonium molybdate. ×120,000.*

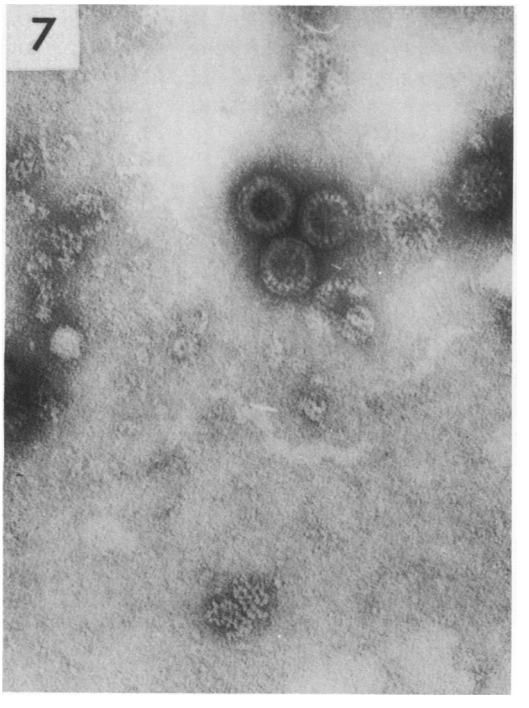

FIG. 7. *Fragmentation of double-shelled particles observed in a preparation of human duovirus after sonic treatment and two cycles of ultracentrifugation. Note that the fragments have a lattice structure, and that no separate capsomers are seen. Ammonium molybdate. ×120,000.*

of calf scours virus. Various other "honeycomb" or latticework structures of irregular shape and different spacings were regularly observed in fecal extracts, both in the presence and absence of duovirus, but these latter are believed to be fragments of bacterial cell walls.

DISCUSSION

On the bases of both morphology and morphogenesis, the new virus described here obviously belongs in the family *Reoviridae*, in which there are at present two recognized genera. It has some features in common with both reoviruses and orbiviruses, notably, the type of viroplasm or "virus factory" produced in infected cells appears identical in each case.

Observation of negatively stained particles alone emphasizes the resemblance of the double-shelled particles to reovirions (15, 17). Never-

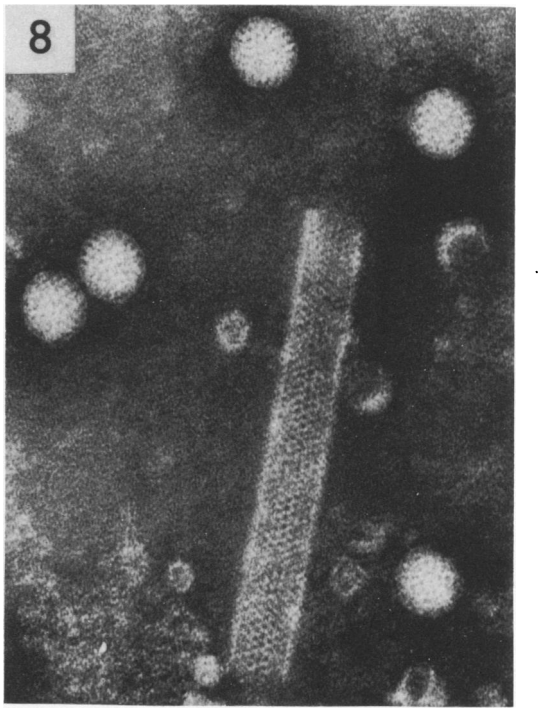

FIG. 8. *Another human fecal extract showing double-shelled virus particles and a flattened tubule with hexagonally packed subunits (10 nm center-to-center spacing). Similar tubules have also been observed in preparations of calf scours virus. ×120,000.*

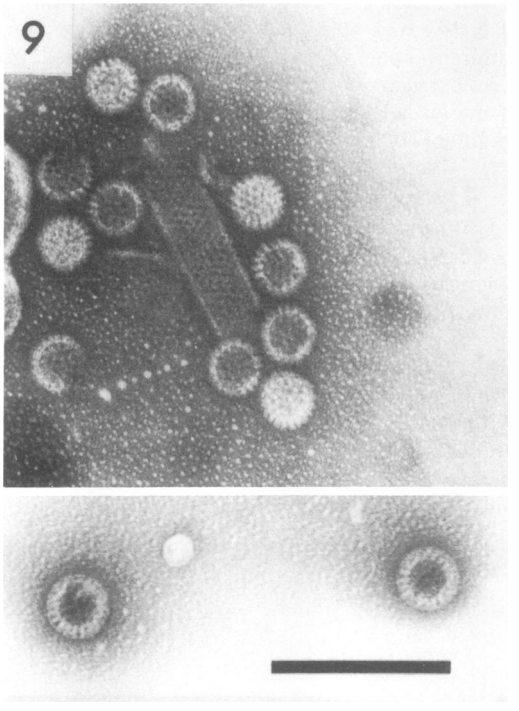

FIG. 9. *A group of EDIM virus particles, mostly single-shelled, and a tubule with the same subunit spacing, 10 nm, as that observed in similar tubules in preparations of human duovirus and calf scours virus. The inset shows two typical double-shelled particles in the same preparation. Ammonium molybdate.* ×*120,000.*

theless the particles of the infantile diarrhoea virus are distinguishable from those of reovirus by their sharply defined outline (7, 16). This feature is notable also in published micrographs of Nebraska calf scours virus (14) and two South African viruses not known to be associated with gastroenteritis, SA-11 and the "O" agent (12). Our crude preparations of EDIM virus also contain double-shelled particles with the same sharply defined outline, although the previously published electron micrographs of purified EDIM virus show apparently single-shelled particles (24).

Single-shelled particles of human duovirus, like those of calf scours virus, EDIM virus, and SA-11, closely resemble orbivirus particles rather than the single-shell "core" particles of reovirus (12, 19, 24, 29). The capsid-related tubules reported above in human stool extracts are almost certainly analogous to those observed within cells infected with EDIM virus (4). In the negatively stained preparations the tubules appear to be flattened, since the alternating clear and fuzzy areas of subunit structure are believed to arise as Moire patterns from superimposition of the upper and lower surfaces, and they extend to near the edge. Similar tubules have been observed both within infected cells and in negatively stained suspensions of several orbiviruses, such as bluetongue, epizootic haemorrhagic disease of deer, and Tribec and African horse sickness viruses (25, 27). It is probable that they are formed by the aberrant assembly of viral capsid material and are analogous to the tubular polyheads of bacteriophage or the tubules found in preparations of various icosahedral viruses (3, 13).

Morphogenesis observed in infected duodenal epithelial cells, in particular the accumulation of enveloped as well as naked particles in rough endoplasmic reticulum vesicles, sets the human duovirus apart from the classical reoviruses and suggests a possible affinity with orbiviruses such as Colorado tick fever virus (26). The same feature has also been reported in morphogenesis of EDIM and SA-11 viruses (1, 18).

The results of our analyses of the RNA and polypeptides of calf and human duoviruses (S. M. Rodger, R. D. Schnagl, and I. H. Holmes, manuscript in preparation) suggest a composition broadly similar but distinguishable from that of either reovirus or orbiviruses. The close serological relationship between the human gastroenteritis virus, "Nebraska" calf scours virus, and EDIM virus which has been established independently by Flewett et al. (16), Kapikian et al. (17), and by our own work, and the impossibility of classifying these viruses as either reoviruses or orbiviruses despite their obvious affinity to both groups, led us to suggest that they should be placed in a new group within the family *Reoviridae*, called "duovirus" (10). The name "rotavirus" has also been put forward for the same group (16).

So far as is known, in vivo growth of each of these viruses occurs only in the highly differentiated cells of gut epithelium. This high degree of tissue specificity no doubt accounts for the difficulty which has been experienced in attempts to grow these viruses in conventional cell cultures. So far only the calf virus has been so adapted (21, 30), but the production of an attenuated oral vaccine for calves (22) is most encouraging and may foreshadow similar developments with the human virus.

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ADDENDUM

During the preparation of this manuscript H. Suzuki and T. Konno (Tohoku J. Exp. Med. **115**:199, 1975) reported reovirus-like particles in jejunal mucosa of a Japanese infant with acute infectious nonbacterial gastroenteritis. One of their micrographs illustrates tubular structures of approximately the same diameter as virus particles in a section of an infected cell.

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