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Electron Microscopic Observations of Visna Virus-Infected Cell Cultures[1]

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Electron microscopic observations of three cell lines infected with visna virus revealed two types of extracellular particles. The smaller of these was 65–110 mμ in diameter and contained a 20–30 mμ electron-dense core. Ordered arrays of the latter type of particle occurred rarely in the cytoplasm. After cesium chloride density gradient centrifugation of the virus, the band that contained maximal infectivity was composed of numerous particles with osmiophilic cores similar to those found in infected cell cultures. This finding suggests that such particles represent the infective agent. The second type of extracellular particle was larger (100–140 mμ in diameter), lacked an electron-dense core, and contained material similar in appearance to cellular cytoplasm. This form appeared to develop by budding from the cell surface.

INTRODUCTION

Visna virus produces a progressive neurological disease in sheep characterized by subacute inflammation and demyelination of the brain and spinal cord (Sigurdsson *et al.*, 1957; Sigurdsson and Pálsson, 1958). The disease begins insidiously, progresses gradually, and usually ends in paralysis and death. Months or even years may elapse between the introduction of the virus and the first manifestation of the disease, hence the term "slow-virus" has been used to describe the visna agent (Sigurdsson, 1954; Sigurdsson *et al.*, 1957).

Visna virus was first propagated in primary tissue cultures of sheep choroid plexus (SCP) cells (Sigurdsson *et al.*, 1960), and most studies of the agent have used such cultures. The virus has also been shown to multiply in a continuous line of embryonic bovine trachea (EBTr) cells (Harter *et al.*, 1968). Infected cultures develop characteristic cytopathic changes, beginning with the appearance of refractile spindle cells, followed by the development of large multinucleated giant cells with long stellate processes, and ending in degeneration of the cell sheet. Newly formed virus is first detected after a latent period of 16–20 hours. Titers increase rapidly during the ensuing 16 hours, and virus continues to be produced until the cell sheet shows extensive degeneration 96–120 hours after infection (Thormar, 1963; Harter *et al.*, 1968).

Visna virus antigen accumulates in the cytoplasm, where aggregates in the form of inclusion bodies are found in giant cells late in the course of infection (Harter *et al.*, 1967,

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1968). Acridine orange staining reveals that increased amounts of RNA collect in the cytoplasm of visna-infected cells (Thormar, 1966) and that the inclusions exhibit the staining properties of RNA (Harter *et al.*, 1967). Incorporation of radioactive uridine, but not thymidine, into the virus indicates that the viral nucleic acid is RNA (Harter *et al.*, 1969).

A previous electron microscopic study of visna virus replication in SCP cells showed budding structures on the surface of infected cells and accumulations of extracellular viruslike particles (Thormar, 1961). Intracellular viral forms or precursors were not seen, and it was suggested that the virus develops by budding from the cell membrane. The present communication reports ultrastructural observations of cells infected with visna virus and of virus purified by density gradient centrifugation.

MATERIALS AND METHODS

Cell cultures. SCP cells were prepared from the choroid plexus of domestic Hampshire or Suffolk sheep and serially propagated by methods previously described (Harter and Choppin, 1967). EBTr cells were purchased from the American Cell Type Culture Collection. SV40-transformed lamb choroid plexus (SCP-T) cells were obtained from Drs. K. Takemoto and L. Sturman of the National Institutes of Health. Cell lines were maintained in 250-ml plastic flasks in reinforced Eagle's medium (Bablanian *et al.*, 1965) containing 10% fetal bovine serum.

Virus. Visna virus K485, kindly supplied by Dr. H. Thormar and Dr. P. A. Pálsson, Institute of Experimental Pathology, University of Iceland, was serially propagated in SCP cells. Eighth, twelfth, and fourteenth passage virus containing 0.8–3.4 $\times$ $10^{6.0}$ $TCID_{50}$/ml was stored at $-70°$ and used as stock.

Infection of cells. Monolayers of SCP and EBTr cells grown in 60-mm plastic petri dishes were inoculated at multiplicities of 0.5–2.0 $TCID_{50}$/cell. These low multiplicities were employed so as to prevent the rapid cell fusion and degeneration that occurs when higher virus:cell ratios are used (Harter and Choppin, 1967). Following adsorption for 3 hours at 36°, maintenance medium containing 2% heat-inactivated lamb serum and 2.0 μg/ml amphotericin B was added, and the cultures were incubated at 36° in a humidified atmosphere of 5% carbon dioxide. At intervals of 48, 72, 96, 120, and 144 hours after infection the supernatant from infected cultures was removed, and 0.5% bovine plasma albumin (BSA. Fraction V, Armour Pharmaceutical Co., Kankakee, Illinois) was added. Such material was quick-frozen, stored at $-70°$ and later assayed for infectivity. The remaining attached cells were prepared for electron microscopy. Control monolayers were inoculated with medium containing 0.5% BSA and handled in a similar manner.

Monolayer cultures of SCP-T cells propagated in 250-ml plastic flasks were inoculated at a multiplicity of 2 $TCID_{50}$/cell. Seventy-two hours after inoculation, cells were collected and prepared for electron microscopy.

Assay of infected virus. Viral infectivity was determined as previously described by observation of cytopathic changes in SCP monolayers (Harter and Choppin, 1967).

Density gradient purification of virus. Media from infected cultures were clarified by low speed centrifugation, digested with bovine pancreatic deoxyribonuclease and ribonuclease, and centrifuged in a cesium chloride density gradient in the manner previously described (Harter *et al.*, 1969). The banded material was collected, diluted with 0.1 *M* phosphate buffer and 0.001 *M* EDTA, centrifuged at 159,300 *g* for 3 hours, and prepared for electron microscopy.

Preparation for electron microscopy. The SCP and EBTr cell monolayers were washed with Tyrode's solutions, and, after the addition of 1% glutaraldehyde, were scaped off the plastic and centrifuged. The resulting pellet was fixed for 20 min in 1% glutaraldehyde, washed, postfixed for 20 min with osmium tetroxide, dehydrated, and embedded in epoxy resin (Epon 812). SCP-T cells were treated similarly, except that they were fixed in glutaraldehyde for 1 hour and osmium tetroxide for 30 min. The latter fixation was also used in preparation of pelleted density gradient fractions for electron microscopy.

Sections of the embedded cells and virus were stained with uranyl acetate had lead

citrate, and examined in an RCA EMU-3G electron microscope.

RESULTS

Uninfected Cell Cultures

Electron microscopic observations of control cultures of each of the three lines showed fibroblastic cells with similar ultrastructural features. The cells contained large, prominent nuclei with well-defined chromatin. Mitochondria were normal in appearance with intact cristae; the endoplasmic reticulum was highly developed with numerous ribosomes attached to the membranes. No viruslike particles were encountered.

Virus-Infected Cultures

Under the light microscope, the first indication of visna virus infection was the development of refractile spindle-shaped cells. As infection progressed numerous large polykaryocytes formed with long slender processes (Fig. 1).

The most prominent features observed by electron microscopy were crescent-shaped buds arising from the cell membrane, and extracellular particles of two distinct types (Figs. 2–6). These were observed in all three cell lines, were associated both with fibroblasts and polykaryocytes, and increased in number as the cytopathic changes progressed and the titers of infective virus rose (Table 1).

The crescent-shaped buds ranged in size from 100 to 140 mμ at their widest diameter and were bounded by a single osmiophilic membrane (Figs. 2–4). They exhibited a clearly defined coat enclosing a thin electron-dense membranous structure, 5.0–6.0 mμ thick. Many contained material resembling the cellular cytoplasm with clearly visible ribosomes. Although the majority of buds were observed in the process of formation at the cell surface, some appeared to have become detached and were found free as extracellular particles without a central core (Fig. 2).

A second type of extracellular particle was spherical or elliptical and smaller in size, the diameter ranging from 65 to 110 mμ. Most contained a single osmiophilic core 20 to 30 mμ in diameter which was separated from the external limiting membrane by a zone of lesser density. Occasionally an additional membrane immediately surrounding the electron-dense core was seen. Particles with 2 or 3 cores were also observed (Figs. 5 and 6).

In rare instances ordered arrays of intracytoplasmic particles with an electron-dense core enclosed by a single membrane and diffuse peripheral coat were encountered within the cytoplasm of infected cells (Fig.

FIG. 1. Phase contrast photomicrograph of polykaryocytes with elongated cellular processes present in SCP culture 96 hours after visna virus inoculation. ×240.

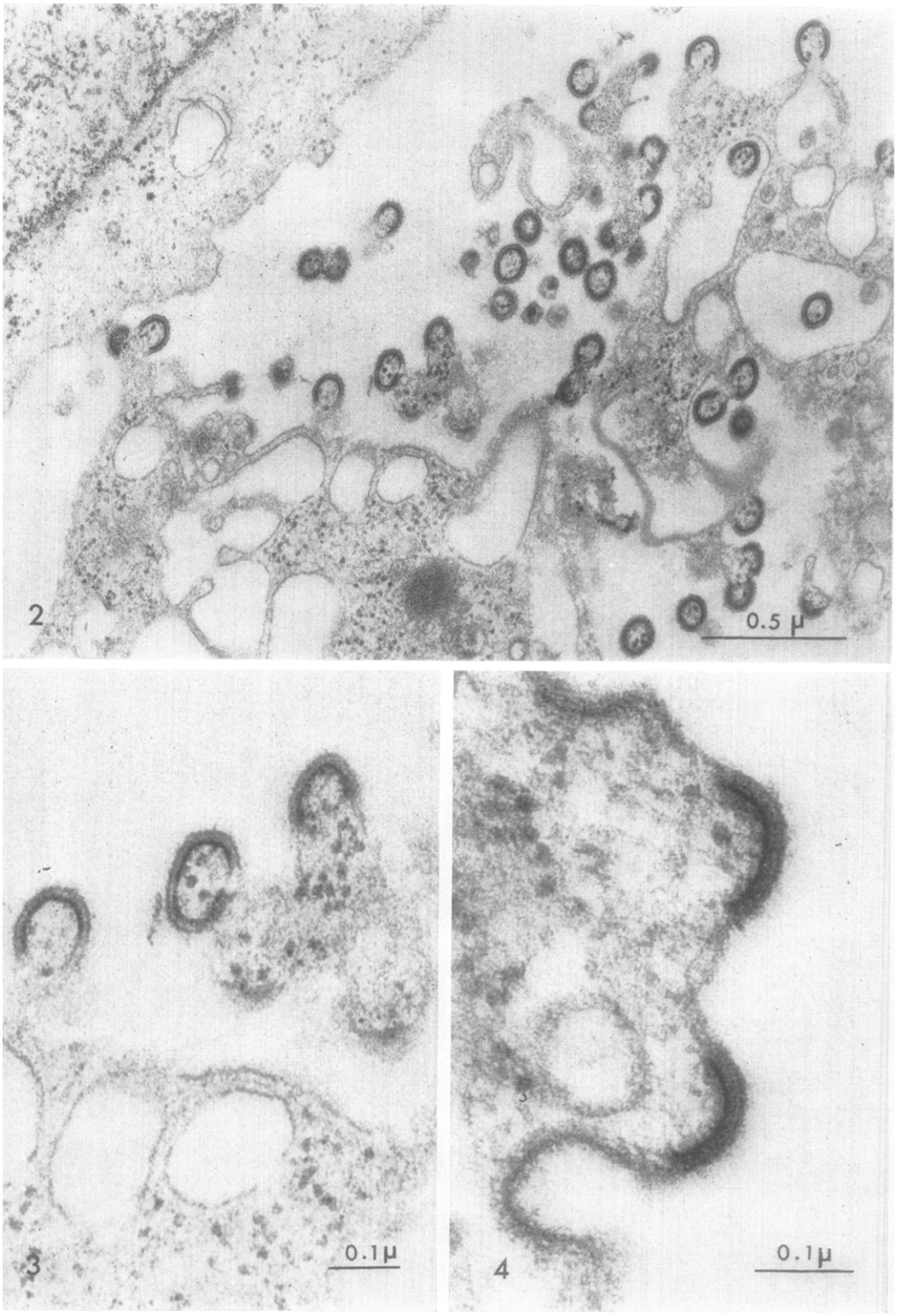

FIG. 2. SCP-T cells 72 hours after inoculation with visna virus, showing buds arising from the cell surface. Some buds are free within the extracellular spaces.

FIG. 3. A higher magnification of buds developing from infected SCP-T cells. Ribosomes are visible within the bud.

FIG. 4. Developing buds in SCP cell 72 hours after infection with virus. The electron dense crescent underlying the bud membrane is shown to consist of a single unit.

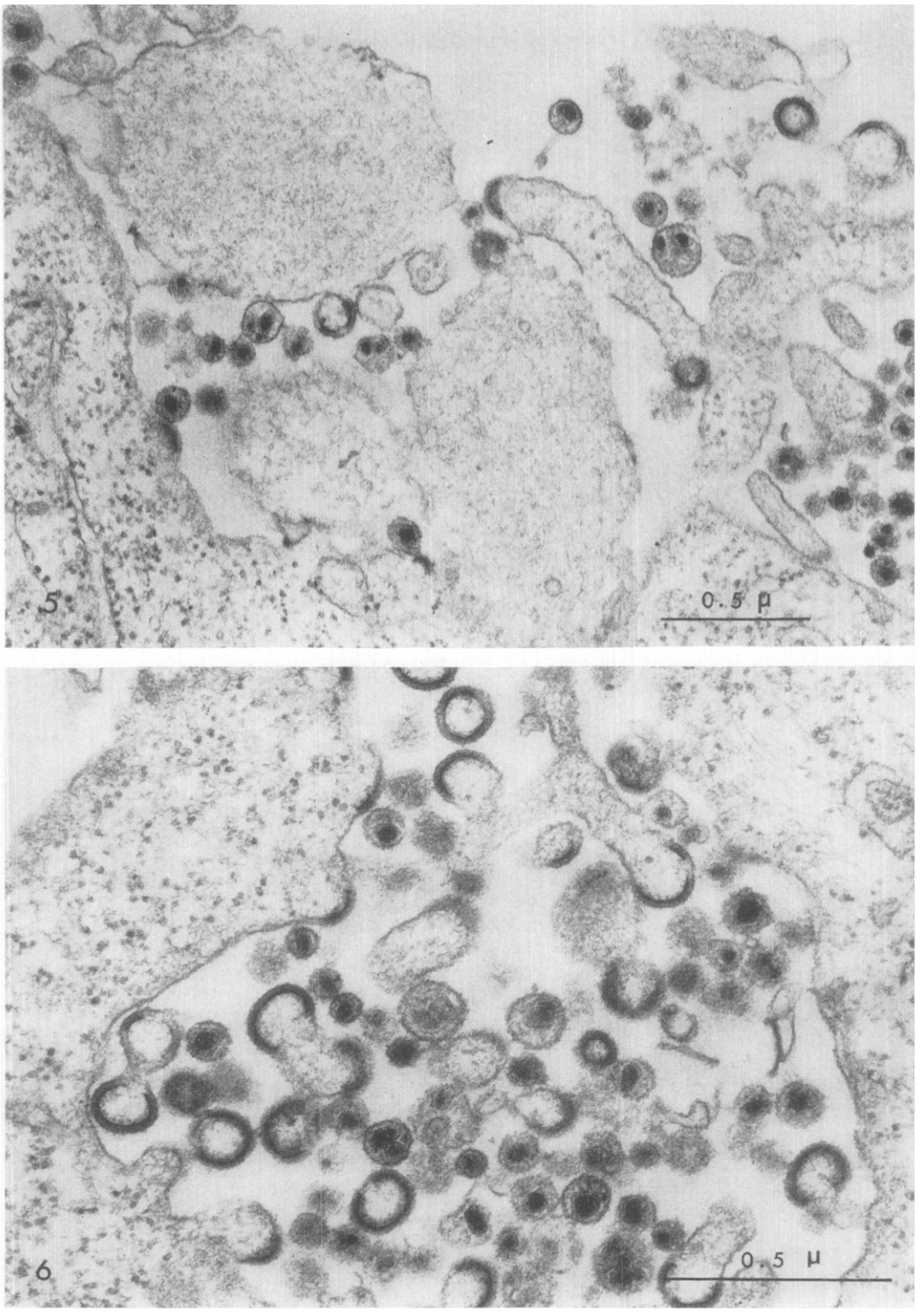

FIG. 5. Extracellular virus in an SCP culture 72 hours post infection. Both single and multicore particles are clearly visible.

FIG. 6. Typical virus accumulation in SCP culture 120 hours postinfection. Many budding structures have detached from cell surface to form particles with translucent centers.

TABLE 1
RELEASE OF INFECTIVE VIRUS IN VISNA-INFECTED SCP AND EBTr CELLS

Time after infection (hours)	Infective virus ($TCID_{50}$/ml)	
	SCP cells	EBTr cells
48	6.3×10^3	2.0×10^4
72	3.6×10^5	4.7×10^4
96	6.3×10^5	—
120	6.3×10^6	—
144	3.4×10^6	—

7). The diameter of these cores was 20–30 mμ and that of the peripheral coat approximated 90–100 mμ.

Viral Particles in Density Gradient Fractions

Density gradient studies using potassium tartrate or cesium chloride have demonstrated that infective visna virus equilibrates at a buoyant density of 1.18 to 1.20 g/ml (Harter and Choppin, 1967; Harter *et al.*, 1969).

Thin sections prepared from the virus obtained after isopycnic gradient centrifugation in cesium chloride revealed a large number of enveloped particles containing single, and occasionally multiple, cores (Fig. 8). These particles appeared structurally to be identical to those with dense cores observed in infected cell cultures as described above.

Structures resembling the detached budding forms were never seen in purified preparations of the virus nor in any other banded fraction obtained by density gradient centrifugation.

DISCUSSION

The presence of two types of extracellular particles in infected cultures raises the question as to which represents the visna virion. Those with electron-dense cores resemble both in size and morphology the particles previously observed in visna virus-infected cell cultures (Thormar, 1961). The finding of many such particles in density gradient fractions containing maximal amounts of infective virus supports the impression that they are the infective agent.

Ordered arrays of intracellular particles have not been previously described in visna virus-infected cells. Crystalline intracytoplasmic aggregates of incomplete or mature virus occur with other RNA viruses, including arboviruses (Morgan *et al.*, 1961; Acheson and Tamm, 1967), picornaviruses (Stuart and Fogh, 1959; Morgan *et al.*, 1959; Fogh and Stuart, 1959), and reoviruses (Tournier and Plisser, 1960). In these systems, however, the accumulations can be related to a specific stage in development of the virus. The collections of cytoplasmic particles in cells infected with visna virus may be associated with the sites of viral assembly, but the infrequency with which such aggregates were encountered makes documentation of this hypothesis difficult.

As already noted, particles lacking dense cores appeared to bud from the cell membrane. It has been suggested that these crescent-shaped buds develop into the particles with electron-dense cores (Thormar, 1961). The present communication does not lend support to this since extensive study failed to reveal transitional forms. It must be admitted, however, that were transition exceedingly rapid the intermediary stages could be so uncommon as to escape notice. Since the particles that lack dense cores and apparently contain cytoplasmic components were not encountered in the density gradient fractions it appears unlikely that they are infectious or represent a helper virus. Rather they may be segments of detached host cell membrane, which has become altered as a result of viral infection so as to resemble the envelope of the buds. A similar phenomenon has been described in the case of herpesvirus infected cells (see Figs. 11–14, 17, and 18 of the paper by Nii *et al.*, 1968). In the latter study it was suggested that when such altered segments of the host cell come into contact with the surface membrane of uninfected cells fusion occurs in a manner similar to that associated with fusion of the viral envelope at the time of entry (Morgan et al., 1968). It is of interest in this connection that visna virus also causes cell fusion (Harter and Choppin, 1967). Possibly the mechanism of cell fusion is analogous.

It has also been suggested that the buds at the surface of visna virus-infected cells resemble those seen on the surface of mouse

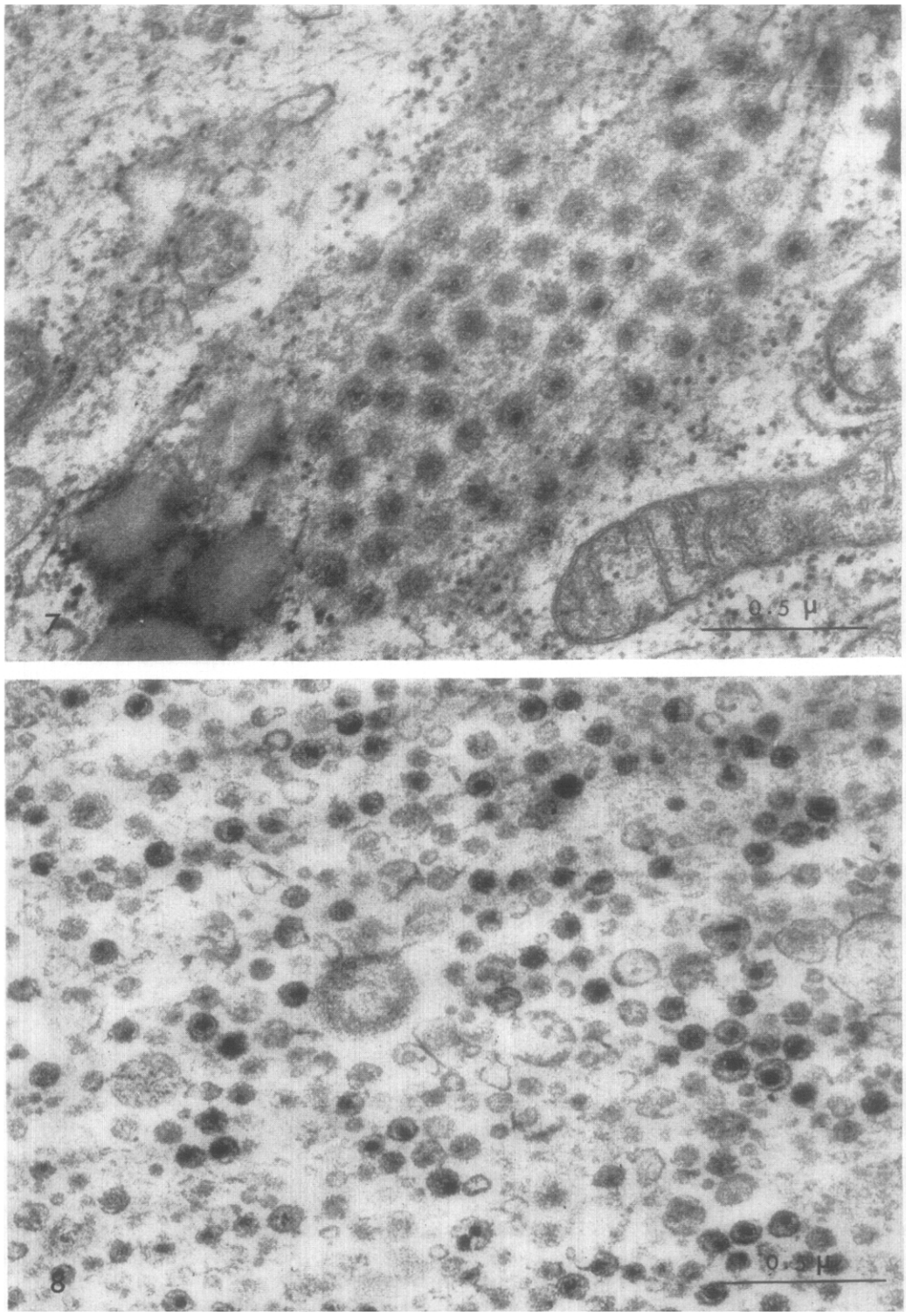

FIG. 7. A crystalline array of virus in the cytoplasm of a SCP cell 120 hours post infection.
FIG. 8. Virus particles in CsCl density gradient fractions containing peak infectivity.

mammary carcinoma cells and mouse leukemia cells in that they develop into double-walled bodies which are released from the cell membrane (Thormar, 1961). With the current improved methods of preparing specimens for electron microscopy, the budding process in visna virus infection appears to differ significantly from that observed in the case of oncogenic RNA viruses. Immature spherical particles, such as those incorporated into budding mammary tumor virus (Lyons and Moore, 1965), were not found. Furthermore, budding forms with an electron-dense central matrix such as those described in studies of the development of murine leukemia viruses (DeHarven and Friend, 1958, 1960, 1966; DeHarven, 1962; Dalton *et al.*, 1961; Dalton, 1966; Ziegel *et al.*, 1966) were never encountered.

It is of interest that cells infected with avian infectious bronchitis virus (IBV) or the related human pathogen, strain 229E, contain two types of viruslike particles, one with an electron-dense core and the other with a translucent center (Hamre *et al.*, 1967; Becker *et al.*, 1967). Which of these represents the infective virus is unclear. Both resemble the particles reported in the present study. Furthermore, buds with a single electron dense layer beneath an outer membrane resembling those seen in visna virus infection have also been encountered within cytoplasmic vacuoles of coronavirus infected cells (Hamre *et al.*, 1967; Becker, et al., 1967). Whether infection with these viruses also results in a specific structural alteration of segments of the host cell membrane remains to be determined.

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