

Bovine Parainfluenza Type 3 Virus Infection: Virus Replication in Bovine Embryonic Cell Cultures and Virion Separation by Rate-Zonal Centrifugation

KIN-SON TSAI¹* AND R. G. THOMSON

Department of Pathology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

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Replicative sequences of a bovine strain of parainfluenza type 3 virus in bovine embryonic kidney and spleen cell cultures were investigated by light and fluorescence microscopy and by ultrathin section and negative-contrast electron microscopy. Observations from light and fluorescence microscopy showed that intracytoplasmic inclusions were detected as small granules surrounding the nuclei of more than 90% of the cell population by day 2 postinoculation. With the increase of postexposure times, these inclusions coalesced into larger bodies which occupied large portions of the cell. Ultrastructurally, the first sign of virus development was the appearance of aggregates of viral nucleocapsids in the vicinity of the nucleus. With the concomitant accumulation of viral nucleocapsids in the cytoplasm, the virus maturation was expressed by budding processes through the cell membrane into round, oval, or elongated forms. Eosinophilic inclusions were demonstrable in many mitotic cells. Ultrastructurally, these cells were observed to produce virus particles by a process identical to that of resting cells. Virions, prepared from infected culture fluid and negatively stained, appeared to be pleomorphic and their diameter ranged from 200 to 600 nm. The virions were separated, by rate-zonal centrifugation, into two subclasses in a sucrose gradient (15 to 60%, wt/wt). The slowly sedimenting virions had a density approximately 1.20 gm/cm³ and an average size of 200 nm in diameter, whereas the faster-sedimenting virions had a density of 1.24 gm/cm³ and average diameter of 400 nm.

Parainfluenza type 3 virus (PI-3V) probably is implicated more often than any other viral agents that have been associated with respiratory disease in cattle. However, the role of PI-3V in the pathogenesis of so-called "shipping fever disease" is uncertain at present, and conflicting views regarding its significance have been advanced (2, 5, 10-12, 23, 24, 28).

In our continuing investigations on various aspects of the pathogenesis of bovine respiratory disease, it has become evident to us that a prime step in determining the significance of the action of the virus lies in obtaining ultrastructural data that concern the functional implication of replication of the virus and the alterations of cells initiated by virus infection. Until recently, only a few reports describing subcellular changes in cells infected with PI-3V had appeared (1, 13, 17, 21). Consequently, more ultrastructural information is needed con-

cerning the effects of PI-3V in its natural host cells and tissues, the bovine species. The present study concerns the development of a bovine strain of PI-3V and its mode of maturation. In addition, ultrastructural aspects of virus formation in mitotic cells and characterization of virions separated by rate-zonal centrifugation are presented. To our knowledge this is the first ultrastructural observation of virus production in mitotic cells that suggests that a persistent infection can be established in PI-3V bovine cell culture systems.

MATERIALS AND METHODS

Virus. A bovine strain of PI-3V was kindly provided by M. Savan, Department of Veterinary Microbiology and Immunology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada. The virus was originally isolated from the nasal swab of a 5-month-old calf (no. 4604-9) that was in a group being studied for shipping fever and had been shipped from western Canada 2 weeks prior to isolation. The isolate was subsequently identified as PI-3V by neu-

¹Present address: Connaught Laboratories Ltd., Willowdale, Ontario, Canada.

tralization and hemagglutination-inhibition tests and immunofluorescence and electron microscopy. The virus stock was prepared by two to four additional passages in bovine embryonic kidney (BEK) cells. For some experiments, virus was concentrated and partially purified by centrifugation. The medium containing released virus was removed from PI-3V-infected BEK cells, and the cellular debris was removed by centrifugation at $800 \times g$ for 10 min. The virus was sedimented at $30,000 \times g$ for 1 h. The resulting pellets were suspended in a small volume of phosphate-buffered saline (pH 7.2) and stored at -70°C . The titers of stocks were in the range of 3.7×10^7 to 2.3×10^9 plaque-forming units per ml.

Cell cultures. BEK and bovine embryonic spleens (BES) obtained from fetuses approximately 2 to 4 months old were used for primary cell cultures. The BEK cells in their second or third passages were used for most of the present work. Madin-Darby bovine kidneys, purchased from Grand Island Biological Co., Grand Island, N.Y., were used occasionally for comparative purposes. All cells were grown as stationary cultures in prescription bottles, Blake bottles, or Leighton tubes, depending on the experiments. Growth medium consisted of Eagle minimum essential medium in Hanks balanced salt solution supplemented with 5 to 10% fetal calf serum, streptomycin sulfate (100 $\mu\text{g}/\text{ml}$), penicillin G potassium (250 IU/ml), and tyrosine (60 $\mu\text{g}/\text{ml}$). For maintaining monolayer cell sheets, the fetal calf serum concentration was reduced to 1%.

Virus infectivity titrations. The virus infectivity was titrated either by cytopathic changes in culture tubes between 4 and 5 days postinoculation or by hemadsorbing activity (4) with calf erythrocytes. The virus infectivity was also titrated on BEK cells by plaque assay, and the virus titers were expressed as plaque-forming units per milliliter.

Growth curves. Monolayers of BEK cells or BES cells were infected with PI-3V at an infectivity titer of 4×10^6 mean tissue culture infective doses per ml, and inocula were absorbed for 1 h at 37°C . Washed, infected cultures were incubated at 37°C and, at appropriate intervals, both culture fluids and cells were harvested from single cultures. Cell-associated and released virus fractions were obtained by a method similar to that of Numazaki and Karzon (19).

Light microscopy and fluorescence microscopy. Cover slips were fixed in Bouin solution at appropriate intervals after infection and were stained with hematoxylin and eosin. For fluorescence microscopy, the direct method was used. The infected and control cell cultures were fixed, at appropriate intervals, in cold acetone, dried in air, and treated for 20 min at room temperature with fluorescein isothiocyanate-conjugated antibody (goat generated), which was purchased from Colorado Serum Laboratory, Denver, Colo. After being washed in three changes of phosphate-buffered saline, the cover slips were mounted in buffered glycerine and examined with a Zeiss fluorescence microscope.

Thin-sectioning electron microscopy. The most extensive series of observations by electron micros-

copy was made on BEK cells infected at an infectivity titer of 4×10^6 mean tissue culture infective doses per ml. Samples of infected cells were harvested at 24, 48, or 72 h after infection. Thin sections of normal and infected monolayers for electron microscopy were prepared as follows. The culture medium was removed from the monolayers and replaced by 20 ml of phosphate-buffered saline. The cells were scraped from the Blake bottles and centrifuged at $800 \times g$ for 20 min. The pellets were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.3) for 1 h. After fixation, 0.2 M sucrose-0.2 M phosphate buffer (pH 7.3) was added to the pellets and left overnight at 4°C . The pellets were cut into approximately 1-mm³ blocks and postfixed in 1% osmium tetroxide in phosphate buffer (pH 7.3) for 1 h. After dehydration in graded acetone solutions, they were embedded in epoxy resin that had been polymerized by incubation for approximately 12 h each at 37°C and 45°C and for 16 h at 60°C . Sections were cut with either glass or diamond knives and stained with uranyl acetate and lead citrate. The specimens were examined with a Philips EM 200 electron microscope.

Negative-staining electron microscopy. Fluid from infected cultures was examined for the presence of released virus as follows. The fluid, after clarification, was centrifuged at $30,000 \times g$ for 1 h at 4°C , and the resulting pellets were suspended in a small amount of phosphate-buffered saline. Some preparations were dialyzed against distilled water for 1 h. A droplet of the suspension was mixed with an equal volume of 1% phosphotungstic acid, pH 6.8. The mixture was removed by a filter paper, and the grid was left to air dry before examination in the electron microscope.

Rate-zonal centrifugation of virions. All rate-zonal centrifugations were performed in a Spinco SW41 rotor at 20°C . For analysis of virions, 2.5 ml each of 15, 30, 45, and 60% (wt/wt) sucrose in a buffer containing 5×10^{-3} M tris(hydroxymethyl)amino-methane-hydrochloride, 10^{-3} M ethylenediaminetetraacetic acid, and 10^{-2} M NaCl (pH 7.4) (TEN buffer) was layered in a centrifuge tube and left overnight at room temperature. A 1-ml volume of virus suspension in TEN buffer was placed on the gradient and centrifuged at $200,000 \times g$ for 40 min. The density of virus fractions was calibrated by lambda pipettes (100 μl) standardized against water.

RESULTS

Growth of virus. Both BEK and BES were productive for PI-3V replication (Fig. 1A and B). However, BEK cells made slightly more virus than BES cells. The infectivity titer of released virus was consistently higher than that of cell-associated virus. The virus titer reached a plateau between 2 and 3 days in both cell systems.

Ultrastructure of cells before virus inoculation. The epithelial cells in our culture systems were examined ultrastructurally. In brief,

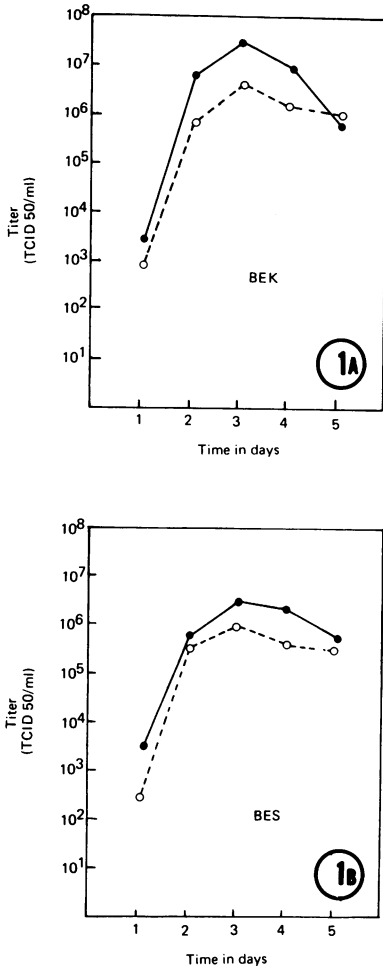


FIG. 1. Growth of PI-3V in BEK cells (A) and in BES cells (B). Symbols: ○, cell-associated virus; ●, released virus.

the nuclei were oval or irregular in shape, with infoldings of the nuclear envelope. The nucleus was composed of rather homogeneously distributed chromatin with a few aggregations along the nuclear envelope and in the nucleoplasm. One or two nucleoli were visible in a single section.

In the cytoplasm, the rough endoplasmic reticulum was distributed in the form of flattened cisternae. The Golgi complex was generally prominent with flattened cisternae and associated vesicles. A few membrane-bound bodies of high electron density, presumably lysosomes, were usually observed in the cytoplasm. Mitochondria were small and numerous; they contained moderately electron-dense matrices and transversely oriented cristae.

Small vesicles, ribosomes, glycogen particles, and bundles of fine fibrils, 7 to 9 nm in diameter, were found randomly scattered in the cytoplasm.

Light microscopy of cells after virus infection. The most significant feature seen in the early infected cultures (1 to 2 days) stained with hematoxylin and eosin was the presence of small eosinophilic inclusions in the cytoplasm (Fig. 2A). By fluorescence microscopy, these intracytoplasmic inclusions were detected as small granules surrounding the nuclei in more than 90% of cell population by day 2 postinoculation (Fig. 3A). With the increase of postexposure times, these inclusions coalesced into larger bodies, which occupied large portions of the cell (Fig. 2B, 3B).

Electron microscopy of cells after virus infection: (i) Early cell alterations. The earliest viral-induced alterations in BEK cells were seen about 24 h after the initiation of infection. At this stage, the most obvious cytoplasmic alterations included a marked increase in the number of free ribosomes, an accumulation of glycogen particles, and a dilated rough endoplasmic reticulum with a low electron-dense content. In some cells, multicentric stocks of Golgi cisternae and scattered vesicular bodies of both low and high electron density were observed.

At about the same time, aggregates of filamentous structures were observed in the vicinity of the nucleus. These filaments, measuring about 16 to 18 nm in diameter, were similar in appearance to the filamentous structures that have been described in cells infected with viruses of the parainfluenza-Newcastle disease-measles group and generally termed viral nucleocapsids (6, 8, 9, 16, 18). Extensive accumulations of nucleocapsids were occasionally seen in the cytoplasm of cells infected with PI-3V at 24 h, but this was more frequent and marked by 48 and 72 h. In some instances, masses of nucleocapsids were observed filling a large portion of the cytoplasm of infected cells (Fig. 4A).

(ii) Viral assembly and release. Although virus buddings were observed in cells by 24 and 48 h after infection, numerous budding processes of virus particles were noted at the plasma membrane of BEK cells by 72 h (Fig. 4A). These buds developed into round, oval, or elongated forms, either free from the cell body or in the process of protruding from the cell membrane (Fig. 4B, C). The diameters of the round particles and elongated forms ranged from 120 to 200 nm, and both types had a unit membrane with an external coating that was considered to

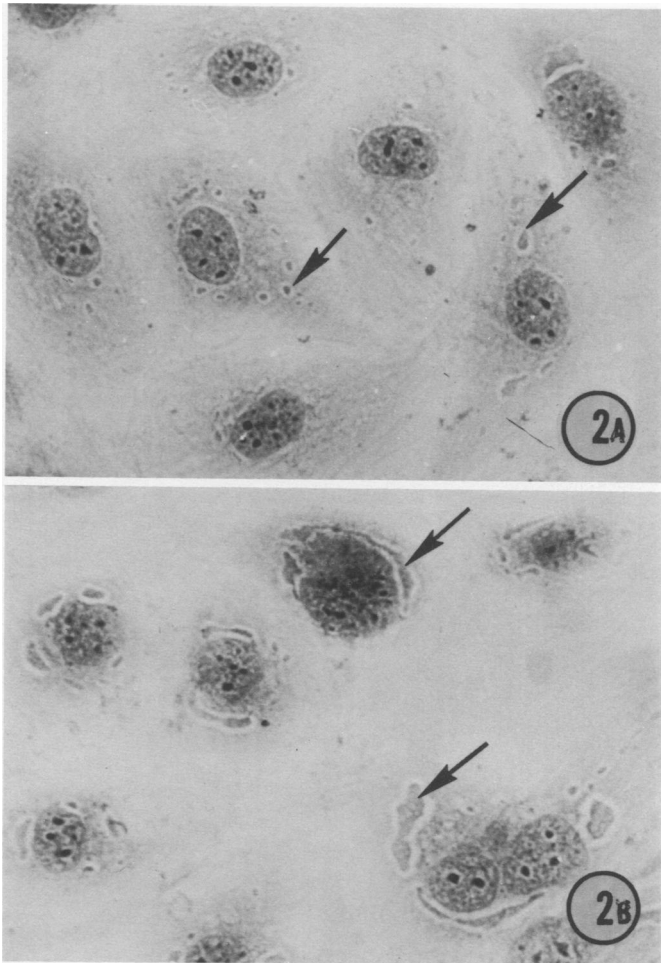


FIG. 2. Light micrograph of BEK cells infected with PI-3V. (A) Twenty-four hours after virus inoculation. Note granular inclusions (arrows) in the cytoplasm. (B) Seventy-two hours after virus inoculation the inclusions coalesced into larger bodies (arrows). Hematoxylin and eosin. $\times 1,400$.

correspond to the spike projections (Fig. 4B, C). Beneath the viral envelope lay the nucleocapsids which, in cross-section, appeared as a hollowed ring structure with the diameter ranging from 16 to 18 nm. In some instances, the nucleocapsid ribbons were seen parallel to the viral envelope.

Two round forms of virus particles, light (L) and dark (D), were usually distinguishable by the arrangement and concentration of nucleocapsids within their envelopes and by the electron density of the entire body. The L particles usually had a regular distribution of nucleocapsid profiles immediately beneath the membrane envelope and an electron-translucent center. Possibly, some of the L particles were the cross-sections of the elongated forms.

The D particles had tightly packed, randomly coiled electron-dense nucleocapsids and were varied in size but generally were larger than the L particles. Frequently, D particles were found to be enclosed within vesicles of varying size in the cytoplasm of cells that appeared to be in the advanced stage of infection (Fig. 5).

Virus infection in mitotic cells. Intracytoplasmic inclusion bodies were observed with a light microscope in many mitotic cells (Fig. 6A-C). These inclusions were irregular, varied in size, and frequently appeared at the two ends of a dividing cell (Fig. 6A, B).

Forty-one mitotic cells were examined ultrastructurally, and the following characteristic features were commonly observed. (i) These cells contained aggregates of viral nucleocapsids

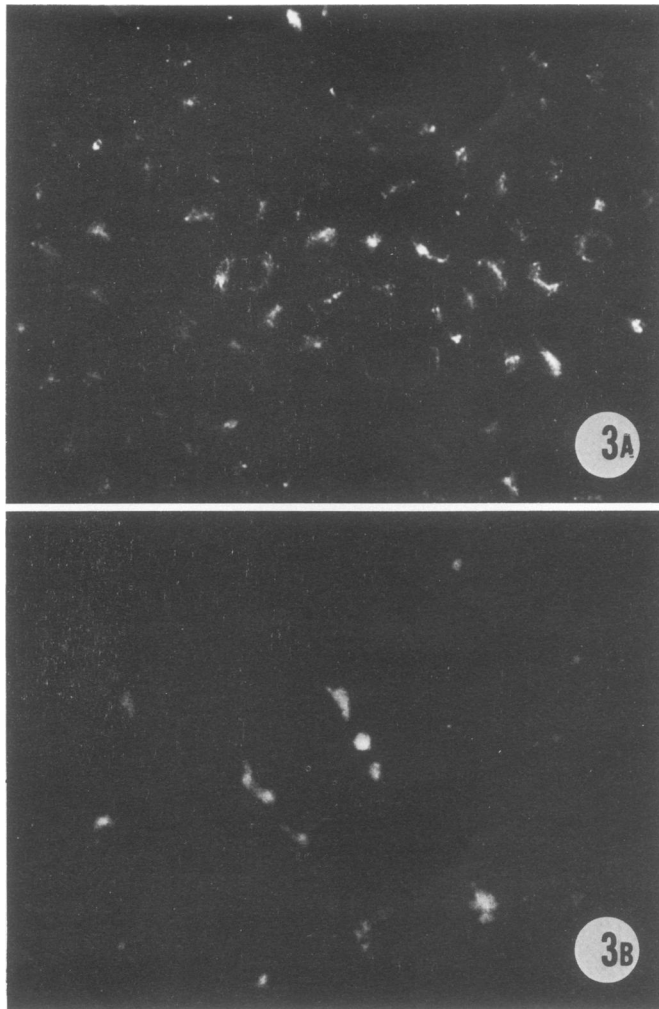


FIG. 3. Fluorescence micrographs of BEK cells infected with PI-3V. (A) Intracytoplasmic inclusions detected as small granules surrounding the nuclei of more than 90% of cell population by day 2 postinoculation. (B) Larger inclusions seen at day 3 postinoculation. (A) $\times 360$; (B) $\times 1,400$.

that were often scattered in the cytoplasm but not associated with chromatin materials. (ii) Some cells were actively producing virus particles by a process identical to those of resting cells (Fig. 4A, 6D). Numerous virus budding forms with various shapes and sizes projected from a single mitotic cell in a given section (Fig. 6D). (iii) When organelles, including the mitochondria, rough endoplasmic reticulum, Golgi complex, and general cytoplasmic matrix, were compared with those of cells without virus infection, it appeared that these mitotic cells were not degenerating by fine structure criteria (Fig. 7).

Virus structure and virions from rate-

zonal centrifugation preparations. Negative-contrast preparations of the resuspended pellets from infected culture fluid contained pleomorphic, round, oval, or irregularly shaped virions, ranging from 200 to 600 nm in diameter. A cluster of helical nucleocapsids was seen within intact or partially ruptured particles, which may result from the physical damage of centrifugation. The negatively stained nucleocapsids were about 18 nm in diameter and had the characteristic features described for other paramyxoviruses (27).

The observations of small-sized virions from previous viral preparations led us to question whether they might represent "incomplete" or

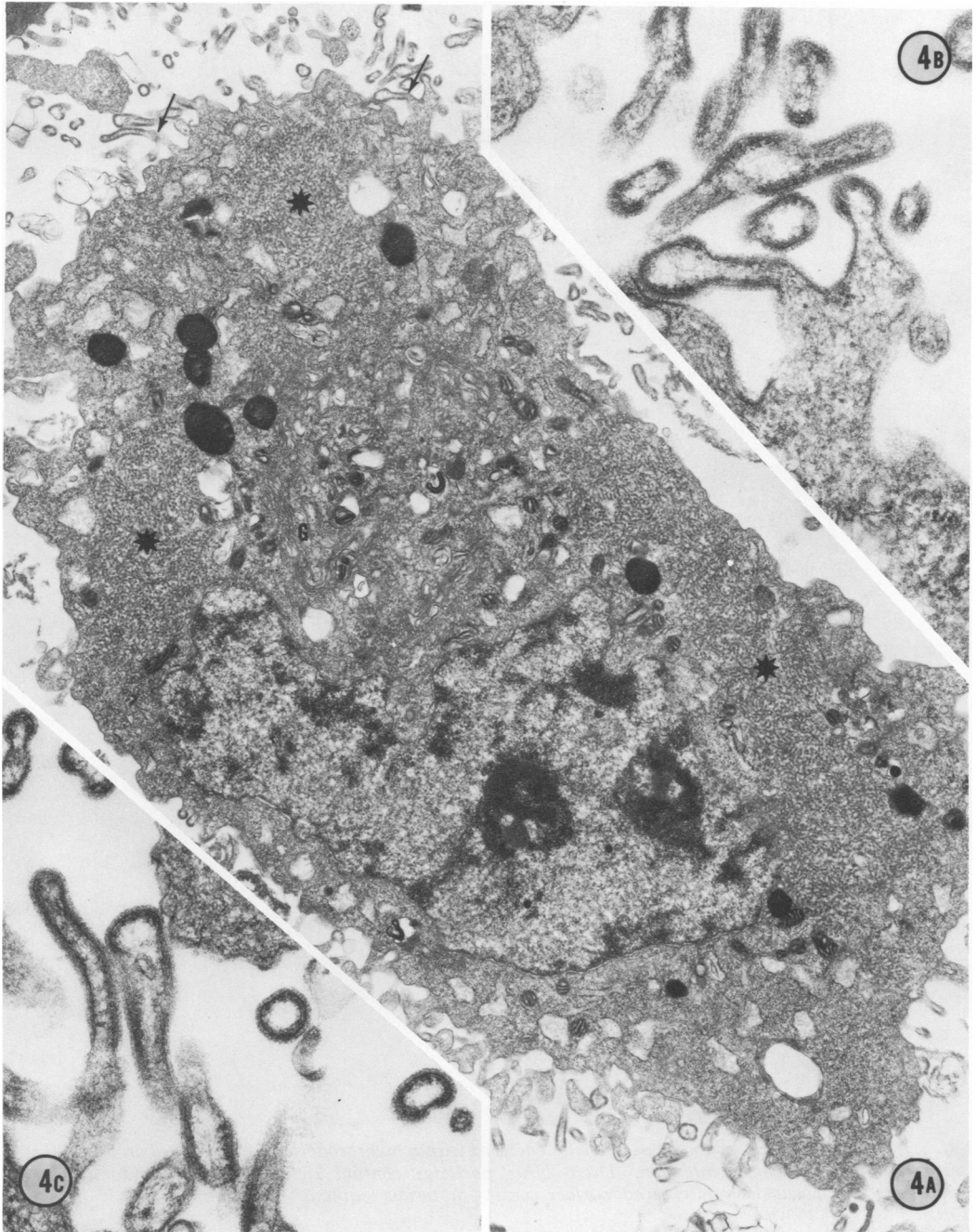


FIG. 4. Electron micrographs of an infected cell. (A) Note the extensive accumulations of nucleocapsids filling large portions of the cytoplasm (stars), Golgi complex (G), and the virus buddings at the cell surface (arrows). (B, C) Enlarged portions from A. (A) $\times 11,450$; (B, C) $\times 33,100$.

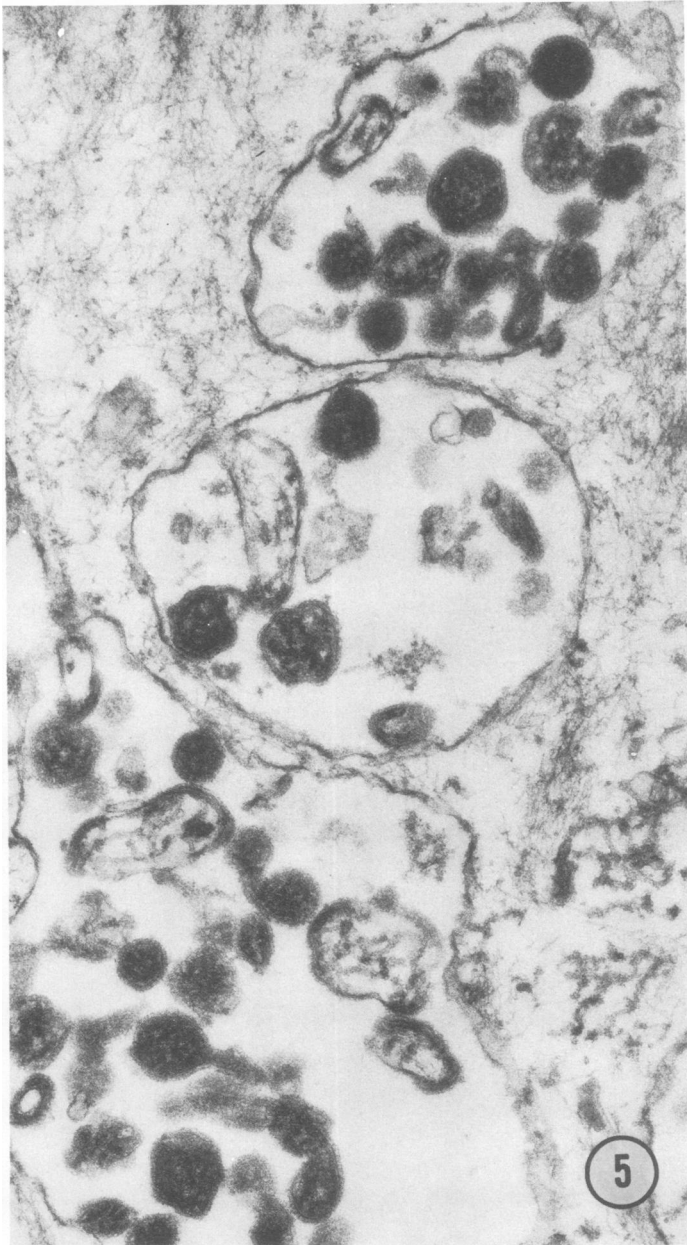


FIG. 5. Aggregates of dark, round virus particles enclosed within membrane-bound vesicles of an infected cell showing advanced stage of infection. These virus particles contain tightly packed, randomly coiled electron-dense nucleocapsids and external surface coating materials (spike projections). $\times 37,500$.

“defective” virus particles. Our preliminary results by rate-zonal centrifugation indicated that at least two classes of virions were present in the viral preparations. Virions with a density of approximately 1.20 to 1.24 g/cm^3 were isolated separately from sucrose gradients and

recentrifuged. Two such cycles of recentrifugation were necessary to obtain reasonably homogeneously sedimenting populations of virions.

Electron microscope examination by a negative-staining method showed that the slowly sedimenting virions had an average diameter of

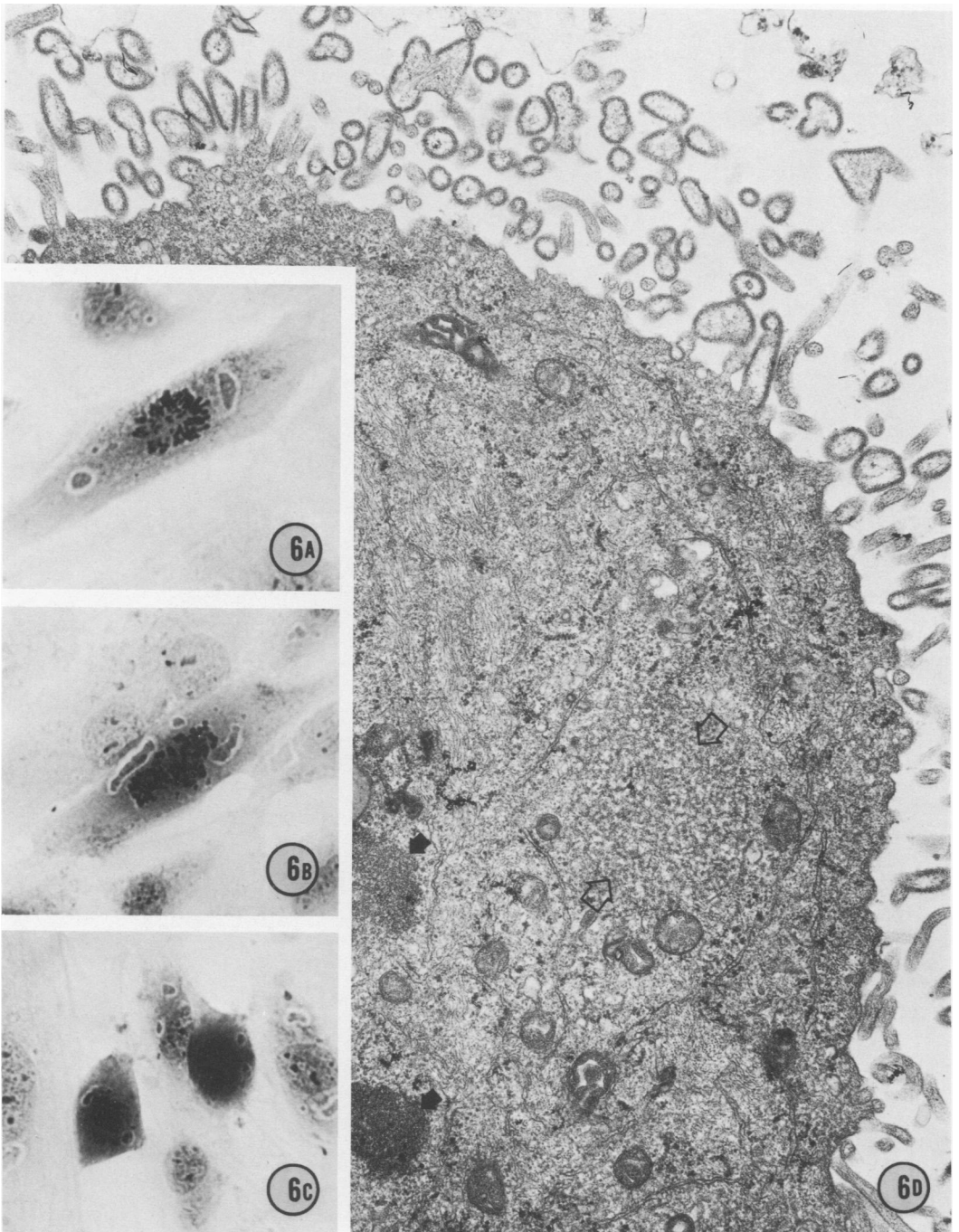


FIG. 6. (A, B, C) Light micrographs of infected cells in mitosis, showing viral inclusions varied in size and shape and frequently at the two ends of a dividing cell. (D) Electron micrograph of an infected cell in mitosis. Note the virus buddings at the cell surface, an aggregate of viral nucleocapsids (empty arrows), and the chromatin substances of portions of isolated chromosomes (solid arrows). (A, B, C) $\times 1,100$; (D) $\times 24,600$.

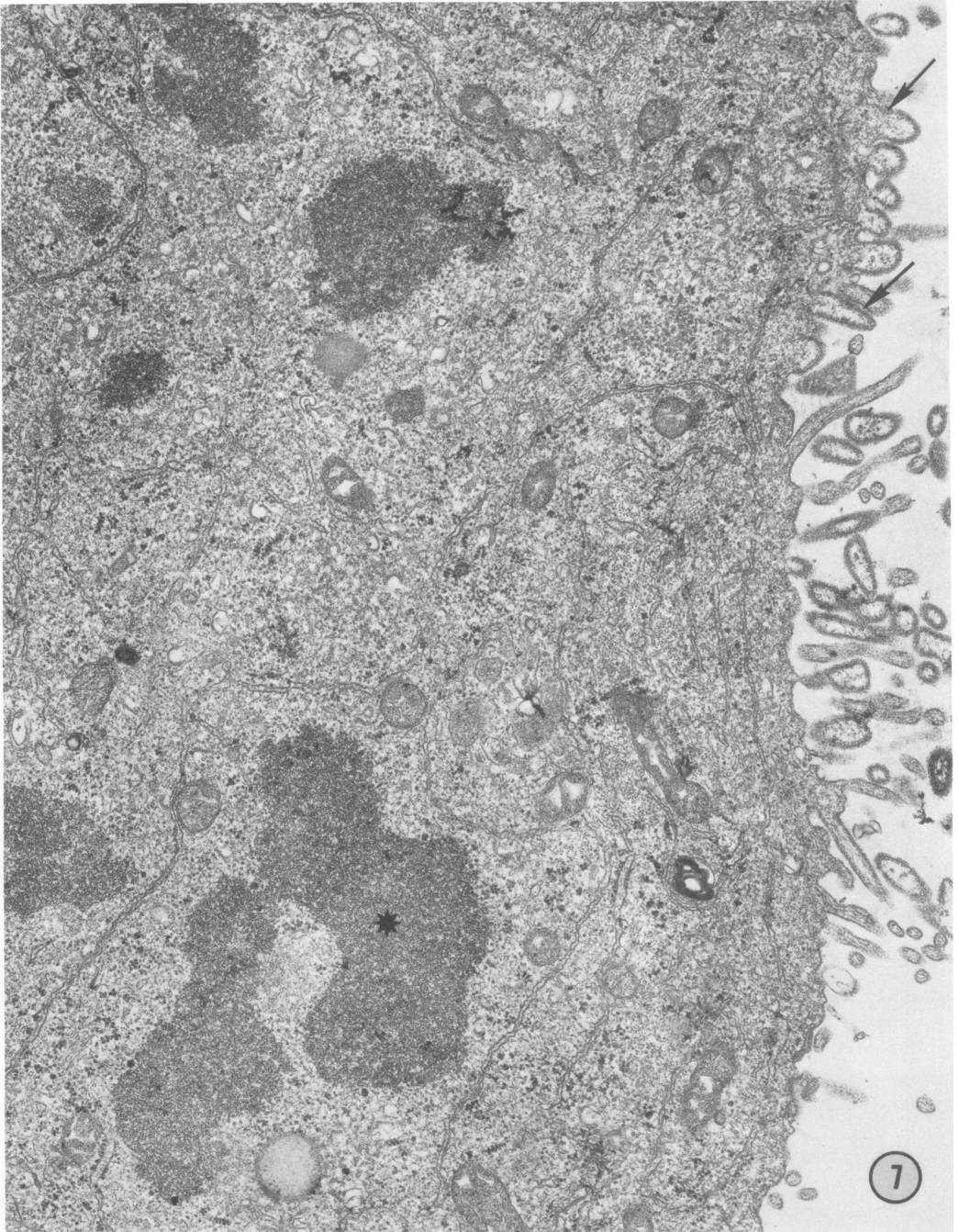


FIG. 7. *Electron micrograph of an infected cell in mitosis. Note the active virus buddings at the cell surface (arrows) and the chromatin materials of randomly sectioned chromosomes, one of which has a typical appearance of a telocentric chromosome (star). $\times 24,300$.*

200 nm, and virions sedimenting to the middle of sucrose gradients had an average diameter of 400 nm (Fig. 8). Virions from the bottom sediment were pleomorphic but generally ranged from 400 to 600 nm in diameter.

DISCUSSION

One of the aims of the present study is to establish a set of ultrastructural data that concern aspects of PI-3V infection in a permissive cell culture system of bovine origin. These data will provide a basis for subsequent investigations on the pathogenesis of PI-3V infection in the bovine respiratory tract and on the interaction between PI-3V and alveolar macrophages.

The term "viral morphogenesis" has often been used by morphological virologists to describe a sequential development and maturation of an assigned virus. In this regard, many excellent papers have been published on paramyxoviruses. Our ultrastructural observations on the viral morphogenesis of PI-3V are comparable to those for other paramyxoviruses, which include Newcastle disease virus (8), simian virus 5 (6), parainfluenza type II virus (16), and measles virus (18). The intranuclear inclusions observed in our BEK cells infected with PI-3V support the view that the intranuclear nucleocapsid formation suggests a terminal stage of virus infection (20). Since there is no direct evidence that intranuclear tubules participate in the process of viral budding or morphogenesis of measles (18), it is difficult to accept the conclusion made by others (17), based on the intranuclear nucleocapsids, that "the morphogenesis of bovine parainfluenza 3 virus more closely resembles that of the serologically unrelated measles virus than that of serologically related parainfluenza virus." In addition, long filamentous virus forms were frequently observed in our PI-3V-infected cell cultures, whereas Nakai et al. (18) reported that "no long filamentous budding particles were observed" in their cell culture system infected with measles virus.

It is apparent that factors from virions (infectivity titer, virulent or attenuated) as well as from cells (species, cell type, primary culture or established line) could influence the distribution of nucleocapsids, the budding activity, and the yield of the virus (7, 8, 14).

Results from the virus growth curve in addition to the present ultrastructural findings indicate that, with diluted virus inoculum (10^4 mean tissue culture infective doses per ml), a productive infection usually occurred in the bovine embryonic cell cultures. Infectious virus

particles were demonstrated as released and cell-associated forms.

In the present ultrastructural study, the dark round particles are probably detached but remained as cell-associated virions. Although similar dark particles enclosed within membrane-bound vesicles were seldom described (6, 8, 16), their frequent appearance in the present cell culture system suggests that they probably represent cell-associated forms of PI-3V. It is conceivable that, once virus particles are actually detached from the cell surface, they are no longer topographically related to the cell body. Thus, considerable numbers of released particles may be no longer demonstrable in the ultrathin sections under ordinary procedures. Only those membrane-enclosed virus particles or particles trapped by the network of cytoplasmic processes or microvilli are likely preserved in the ultrathin sections.

In ultrathin sections, large numbers of the filamentous virus forms were observed in the infected BEK cells. They are assumed to be a transitional form in the process of virus release in the present study. This assumption is contradictory to the generally accepted ideas that the elongated viral elements are another form of paramyxovirus and that they may be attenuated (3, 9) or more virulent (8) forms. Although it was not possible to determine whether any of the light round forms that lay separate from the cells were actually continuous with nearby cell membranes, some of them, at least, are considered to be cross-sectioned forms of filamentous viral elements. Investigations by others on the virus-cell interaction of a parainfluenza virus, simian virus 5, indicated that filamentous forms were observed only in the ultrathin sections and were usually not seen in negative-staining preparations (6). In studies on the morphogenesis of Newcastle disease virus in chicken embryo, Donnelly and Yunis (8) also found that pellets from Newcastle disease virus-infected chorioallantoic fluid contained mostly the dark, round, or ovoid virus forms. None of the virus particles was typically filamentous or as long as those found in the sections. Howe et al. (16) also stated that filamentous forms were never encountered in their negatively stained preparation of type 2 parainfluenza virus. Thus, several possibilities with regard to virus budding and maturation appear to be deducible from previous findings by other (1, 8, 16) and our present observations. (i) The filamentous forms with regularly spaced nucleocapsids beneath the viral envelopes observed in fixed ultrathin sections are not entirely separated

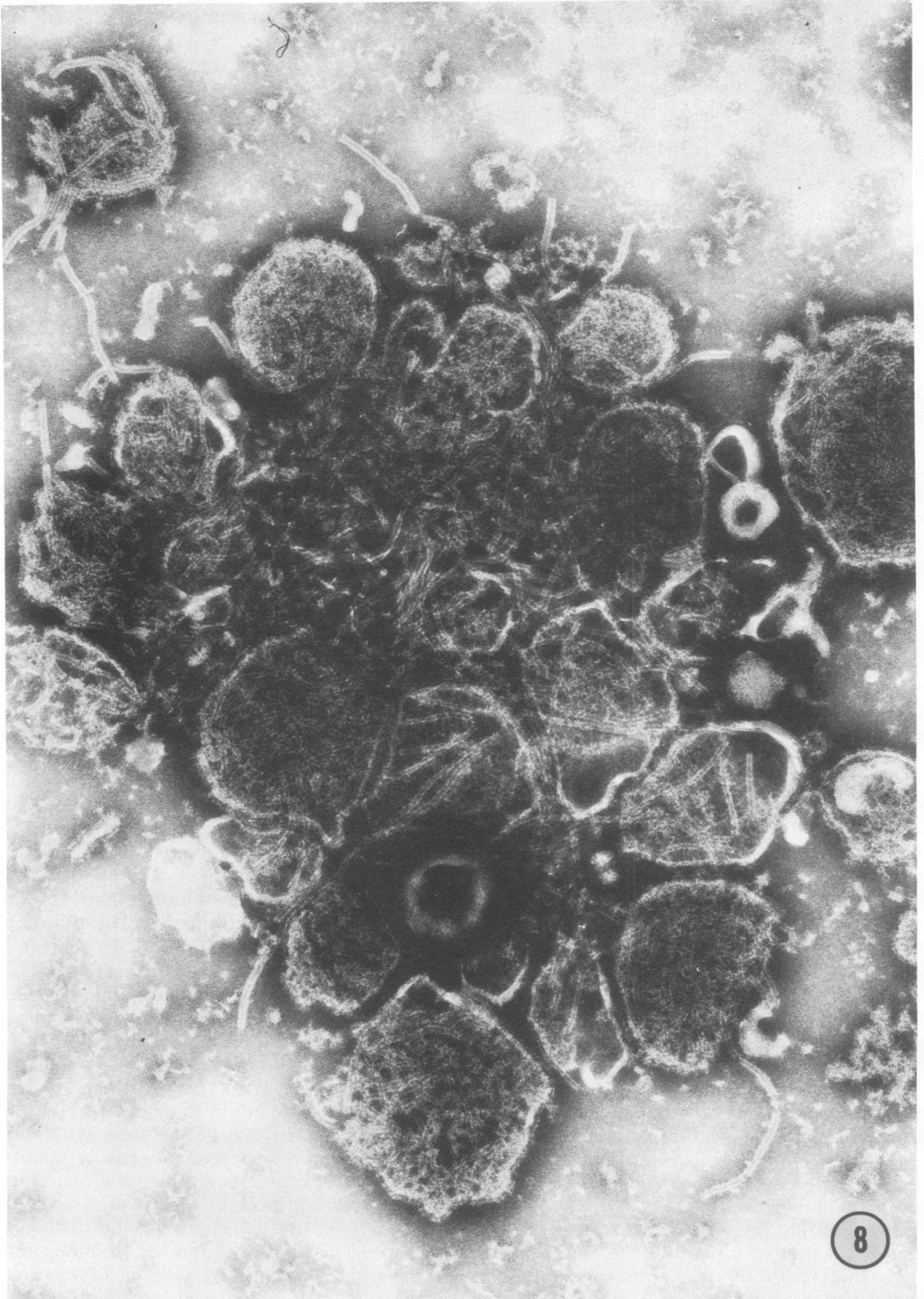


FIG. 8. Negatively stained PI-V3 prepared from rate-zonal centrifugation. The virions contain coiled nucleocapsids and surface or spike projections and have average diameters of 400 nm. $\times 69,300$.

from the cell body and represent transitional forms of virus budding in action. (ii) These elongated forms transform into spherical types when eventually pinched off into culture medium or later in the unfixed buffer solutions used for negative staining. (iii) The previous regularly arranged nucleocapsids become randomly distributed within the virus envelope. (iv) The size of these spherical or irregularly shaped particles in unfixed solutions depends upon the length of the filamentous forms when they finally pinch off from the cell surface. (v) If these viral elements pinch off when they are too short, with less than an adequate amount of viral genome, incomplete or defective virus particles may result.

At the ultrastructural level, our data provided the first morphological evidence that mitotic cells infected with PI-3V are actively engaging in the production of virus particles. In some infected mitotic cells, despite the presence of aggregates of nucleocapsids in the cytoplasm and virus buddings at the cell surface, these cells do not appear to be degenerating by ordinary ultrastructural criteria. The present observations suggest that PI-3V can infect BES cells, replicate in cells, and transmit the virus genome to daughter cells during cell division without the virus going through a surface transmission.

Persistent infections of cells in culture have been established with a variety of lytic viruses (25). These interactions are characterized by detection of plaque-forming virus, accompanied by cell destruction or a continual production of virus without massive destruction of cells. Mumps virus infection of human conjunctiva cells resulted in infectious progeny and cell destruction. If, however, the growth conditions for these cells were such that cells were able to divide, a persistent infection was found (26). In animals, persistent or chronic viral infections result in occasional bursts of detectable infectious virus as well as in continuous production of virus (15, 22).

Although two density classes of PI-3 virions were separated by rate-zonal centrifugation in the present study, the functional significance of the slowly sedimenting virions, which had a density approximately 1.20 mg/cm³, remains to be determined by further investigation.

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