# The Morphogenesis of Virulent Newcastle Disease Virus in the Chick Embryo

## *An Ultrastructural Study*

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NEWCASTLE DISEASE VIRUS (NDV) is a paramyxovirus widely used in the study of interferon production *in vivo*.[1–4] Although it largely afflicts avian species and does not usually reproduce in mice or rabbits, a recent report indicated that NDV multiplied in the spleens of mice previously infected with mouse cytomegalovirus.[5] This prompted our ultrastructural study of NDV multiplication in permissive and nonpermissive living animal host systems in order to ascertain the reactions of different types of cells to paramyxovirus infection. Knowledge of NDV morphogenesis in these systems may be useful in clarifying the effects of interferon and virus interference on virus replication in living animal hosts. This report concerns NDV replication in a natural permissive host, the chick embryo.

Few ultrastructural studies of NDV assembly in any system *in vivo* have been reported and all concern the chorioallantoic membrane (CAM) of the chick embryo.[6–9] Bang studied the morphogenesis of the virulent CG, chicken avirulent and Blacksburg vaccine (Bl) strains of NDV.[6,7] He reported filamentous NDV formation in the CAM and in whole mounts of CAM explant cultures fixed in osmium vapor.[10] These studies formed the basis for his suggestions that avirulent NDV multiplied in the surface area of the cells and was possibly released from the ballooned tips of modified microvilli while the virulent strains were released with the death of the cell. He further suggested that filamentous forms of NDV tended to develop mainly on cells that either had normal microvilli, such as allantoic epithelium, or that had the capacity to form microvillous projections.[6,7]

Blough briefly described the morphogenesis of the Milano strain of NDV in the CAM [11] and reported that it resembled the development of the CG strain. He also described greater production of filamentous

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forms of NDV on the allantoic epithelium of chick embryos that had been treated with vitamin A alcohol intra-allantoically 6 hours after infection with the Milano strain. He stated that the overall morphogenesis of that strain, under the latter circumstances, was more comparable to that of the vaccine (Bl) strain described by Bang.[6]

Our preliminary study [8] of the CAM of chick embryos infected with the CG strain, yielded data not in agreement with the previous reports [6,7] and suggested that certain generalizations [11] about the formation of filamentous forms of NDV were not correct. We found that the mesodermal rather than the allantoic layer was the main site of replication of the CG strain of NDV in the CAM. An important feature was the widespread production of round budding viral forms and characteristic filamentous forms on the cytoplasmic membrane of mesodermal fibroblasts. Fibrillar inclusions were found in the cytoplasm of several cells.

The present report expands and corroborates our preliminary findings in the CAM. Specific attention is directed to clarifying the mode of NDV assembly and to differentiating normal ultrastructural components of chick embryo tissues, especially microvilli and cytoplasmic fibrils, from virus forms, virus components and cellular changes caused by virus infection.

## Materials and Methods

### Chick Embryos

Chick embryos were obtained from two different flocks known to be free of NDV infection. For the ultrastructural study of NDV infection, fertilized eggs from a local commercial flock were incubated for 10 days by the supplier in a humidified atmosphere at 37 C and automatically turned every 2 hours. They were incubated in the laboratory for an additional 24 hours prior to use. For one ultrastructural control study and for plaque assay of chorioallantoic fluid, fertilized, nonincubated eggs were obtained from a pathogen-free flock (Spafas, Inc, Norwich, Connecticut) known to be free of avian lymphomatosis (CoFAL negative). They were developed into viable 9- and 11-day embryos in the laboratory under the conditions noted above.

### Virus and Assay

The virulent CG strain of NDV was used in all experiments. The seed pool, previously passed in eggs from the same local flock noted above, was maintained at −70 C in chorioallantoic fluid between passages and was rapidly thawed immediately before use. It had an infectivity of $10^{8.5}$ $TCID_{50}$/0.1 ml of chorioallantoic fluid when tested in cultures of baby hamster kidney (BHK-21) cells with epithelial characteristics (over 150 passages) (Microbiological Associates, Baltimore, Maryland).

In this study, the chorioallantoic fluid of moribund embryos had an average infectivity of $5.6 \times 10^8$ plaque forming units/ml in assays performed on pathogen-free chick embryo cell cultures according to the method of Osborn and Medearis [5] using starch overlays.

### NDV Infection

A total of twenty 11-day-old chick embryos were infected via the allantoic sac with 0.1 ml of a $10^{-2}$ dilution of NDV-infected chorioallantoic fluid in the course of four separate studies. Eleven control embryos were either inoculated with 0.1 ml of Hank's balanced salt solution or were not inoculated. All embryos were incubated in a humidified egg incubator at 37 C and were turned regularly. In one study, control and infected embryos were harvested 6, 18, 24, 32 or 36 hours after inoculation. The remaining infected embryos were harvested 32, 36 or 42 hours after inoculation. Inoculated and noninoculated control embryos were harvested at the onset and after 24, 36 and 44 hours.

### Electron Microscopy

Under aseptic conditions, the shell was removed over the air sac and the shell membrane and subjacent attached CAM were excised and fixed immediately. The chorioallantoic fluid was pipetted from the allantoic sac into sterile chilled containers. Pieces of CAM from the other end of the egg, the amnion and portions of embryonic brain, liver, spleen, lung and gut were also excised and placed immediately in cold, phosphate-buffered 2% glutaraldehyde for 1–2 hours. They were then rinsed three times in cold, phosphate-buffered sucrose solution and postfixed in phosphate-buffered 1% osmium tetroxide for 45 minutes at 4 C and 45 minutes at room temperature. In one series, pieces of control and infected tissues were then additionally washed in 0.1 N sodium acetate, fixed for 20 minutes in 0.5% aqueous uranyl acetate at pH 3.9 and then washed again in 0.1 N sodium acetate, according to Terzakis.[12]

To prepare chorioallantoic fluid for thin sections, individual portions of thoroughly mixed fluid from individual experiments and from the seed pool were separately centrifuged at 100,000 *g* in a Spinco Model L centrifuge for 2 hours at 4 C. The supernatant was discarded and the pellet was fixed in glutaraldehyde and osmium tetroxide as above.

All tissues were dehydrated in graded alcohols and propylene oxide and embedded in araldite-Epon-812. One-micron sections of pellets and tissues were stained with toluidine blue. Thin sections were stained with uranyl acetate for 30 minutes and then with lead citrate for 5 minutes.

Negative stains of the chorioallantoic fluid were prepared by resuspending the centrifuged pellet in a small quantity of supernatant and mixing portions of the suspension with 1% phosphotungstic acid according to the method of Parsons.[13]

## Results

### Normal CAM

In the 11- and 13-day-old embryos, the allantoic layer of the CAM was usually two cells thick and varied from one to four cells in thickness. The cells often interlocked and numerous desmosomes with attendant tonofibrils lay along the distinct unit plasma membranes. In addition to mitochondria and endoplasmic reticulum, those cells lining the allantoic cavity contained dark superficial membrane-bound granular bodies of variable electron density (Fig 1). Allantoic cells near the subjacent basement membrane had fewer granules. Pinocytotic vacuoles, microtubules, ribosomes, glycogen granules and groups of bundles of straight

or curving cytoplasmic fibrils, 70–90 Å in diameter, were frequently found. (Fig 1) The latter were comparatively more obvious in the CAM of 13-day-old embryos and in the CAM of 11-day-old embryos that had been postfixed with uranyl acetate. Short, stubby, curved or straight, cylindric microvilli projected into the allantoic cavity from the luminal surface of the epithelium. Their numbers and shapes varied from cell to cell. They ranged from 4000 to 9000 Å in length and from 600 to 1200 Å in width. Ballooned, bulbous, clavate and branched microvilli were common. When sectioned along or across their major axis (Fig 2), microvilli were found to contain cytoplasmic fibrils, 70–90 Å wide. The fibrils were straight and lay toward the centers of the microvilli, well removed from the unit plasma membrane. Dark cells containing occasional myelin figures, or irregularly outlined vacuoles, in addition to normal organelles, were found in the allantoic epithelium. They were smaller and appeared crowded by more normal adjacent cells.

Basement membranes separated the allantoic and chorioallantoic layers from the mesodermal layer. The mesodermal layer contained stellate and fusiform fibroblasts, collagen bundles and variable numbers of blood vessels. Concentric layers of fibroblasts usually surrounded the thick-walled vessels. The fibroblasts had long, slender, occasionally branched cytoplasmic extensions and were rich in endoplasmic reticulum and ribosomes. Pinocytotic vesicles in various stages of formation frequently lay on or near the surface membranes of fibroblasts. Mesodermal cells near the allantoic layer occasionally contained ovoid membrane-bound granular bodies of variable electron density, similar to those in luminal allantoic epithelium. No ribbon-like intracytoplasmic structures were found in fibroblasts comparable to those reported by Borysko and Bang.[14]

The chorion, its blood vessels and their relationship to the shell membrane have been evaluated by light microscopy [15,16] and electron microscopy.[17–19] The chorionic vessels, as noted by Ganote *et al*,[19] were separated from the shell membrane by thin cytoplasmic extensions of chorionic epithelium. In most preparations, the shell membrane was closely applied to the chorionic epithelium. When the shell membranes separated from the CAM during fixation, long microvilli were readily evident on the subjacent chorionic epithelium. They were longer than allantoic microvilli, usually single, and often had slender, centrally located cytoplasmic fibrils. Several membranes contained scattered necrotic cells and debris squeezed between normal chorionic epithelial cells or beneath the overlying shell membranes.

The shell membrane was closely applied to the chorionic epithelium

but frequently small spaces were present between them. Projecting from the outer surface of the membrane into the air sac were numerous ovoid-to-fusiform, dense, granular, hair-like extensions, each with a less dense fusiform central segment.

#### NDV-Infected CAM

In all infected embryos, the mesodermal layer contained the largest numbers of viral particles. In embryos harvested before 32 hours of infection, virus production was less pronounced than in embryos harvested after 36 hours. Although fewer in number, microvilli were still present after 36 hours of infection.

Virus forms did not bud from the tips of microvilli in either the allantoic or chorionic layers. As previously reported,[8] round, ovoid and filamentous viral buds were found on the surface of mesodermal fibroblasts or lying between clusters of cells (Fig 3). The budding process was not confined to specific areas of the cell; buds developed along slender cytoplasmic extensions as well as from the plumper regions. As many as six or eight budding forms in different stages of development, and with various shapes and sizes, projected from a single fibroblast in a given section. As previously illustrated, the buds had a thick, dark unit-membrane envelope beneath which lay round cross-sections of nucleocapsid ribbons. The envelope was continuous with the adjacent cell membrane. The cell membrane near the attachment of several buds was split so that the inner layer was distinctly separated from the outer layer (Fig 4). The fuzzy external coating on the outer layer was thought to correspond to the spikes. Several types of round or elongated viral forms often lay apart from the cells and adjacent to the developing buds (Fig 5). The round bodies ranged from 1200 to 1700 Å in diameter and had an external fuzzy coating. Two types of round bodies, light and dark, were distinguishable by the concentration and array of nucleocapsid material within their envelopes and the electron density of the entire body. The dark bodies usually had tightly packed, randomly coiled electron-dense nucleocapsid strands about 120–150 Å in diameter. The lighter particles had fairly uniform distribution of dense nucleocapsid profiles, 110–150 Å in diameter, immediately beneath the unit-membrane envelope. The number of nucleocapsid profiles in the lighter forms ranged from eight to more than 16. On occasion, the nucleocapsid dots had an electron-translucent center, suggesting that they were tubular.

The filamentous forms were straight, slightly curved or occasionally bent. Because they often did not lie completely within the plane of

section, it was not possible to determine their average length. However, several examples were nearly 3 μ long. Their diameters ranged from 1500 to 1700 Å. Most filamentous virus forms were light, but occasional dark forms were found. The arrangement of nucleocapsid within the filamentous forms varied considerably. Near their attachment to the cell, the nucleocapsid ribbons were often arranged in a helical configuration as previously illustrated. In more distal areas, and depending upon the plane of section, the ribbons often followed a sinuous, swirling or straight pattern. The nucleocapsid was not observed to branch. When filamentous virus buds were sectioned in the long axis close to their centers, the nucleocapsid appeared as a row of round dots, immediately beneath the thickened, spiked unit-membrane envelope. Such examples morphologically resembled the light round particles described above. Tubular membranous structures resembling hollow cores were present in the centers of several filamentous viral forms.

Fibroblasts with early or distinct bud formation at the cell membranes occasionally contained fibrillar cytoplasmic inclusions not surrounded by a unit membrane. These cells often had darkened and distinctly thicker areas of cell membrane. The fibrillar inclusions were more common in embryos with the most extensive anatomic evidence of virus production.

After extensive search, aggregates of tubular material, presumably nucleocapsid, were found in mesodermal fibroblasts (Fig 6). These were comparable to those reported in tissue culture studies of other paramyxoviruses (see below). The involved cells usually had extensive virus budding at their cell membranes. The tubules had an external diameter ranging from 110 to 140 Å, and, although they were more readily seen in those tissues postfixed with uranyl acetate, their demonstration was difficult. The aggregates were not membrane bound and were not confined to particular areas of the cells—*ie,* they were not confined to the juxtanuclear or peripheral regions of the cells. The cytoplasm of several large fibroblasts was almost completely replaced by these tubules. On occasion, the tubules were directly continuous with the denser and more readily seen nucleocapsid profiles of the filamentous or round budding forms. In the buds, the nucleocapsid profiles ranged from 110 to 150 Å in diameter. Cells with the largest aggregates of tubular material also were severely altered in other respects; myelin figures and vacuolated degeneration were common. No comparable tubules were found in any cell nucleus.

Morphologic evidence of NDV production on the allantoic surface was limited to the presence of an occasional bud or filamentous form

(Fig 7). This was true of all specimens examined, including those sacrificed after 6, 19 or 24 hours. With longer incubation, round and filamentous virus was found between epithelial cells, especially in the basilar row. Microvilli and filamentous virus forms had comparable diameters and had to be differentiated on an anatomic basis. Microvilli were occasionally found on infected allantoic epithelial cells. Intracellular changes included focal necrosis, myelin figures and occasional distorted mitochondria. Desmosomes and tonofilaments generally were intact. Microtubules, Golgi apparatus, membrane bodies and pinocytotic vesicles were common. No aggregates of tubular material comparable in size to those seen in fibroblasts were found in allantoic or chorionic epithelium. No intracellular round virus forms were found. The only intracellular membrane-bound bodies were vesicles and granular bodies also noted in the normal CAM. Dark and light round virus forms were not common within the lumens of vessels in the CAM. Occasional buds were found on the luminal side of endothelial cells (Fig 8).

Round and filamentous virus forms were occasionally present in the chorion of extensively infected embryos. They lay between cells and numerous budding forms were present.

#### NDV-Infected Amnion and Spleen

To further characterize the infection of chick embryo by NDV, the amnion and spleen were also studied. The mesodermal layers of the amnion had extensive virus proliferation comparable to that noted in the CAM. Epithelial necrosis was extensive. Dark fibrillar intracytoplasmic inclusions were more common in the amnionic fibroblasts than in those of the CAM.

The chick embryo spleen contained more virus forms per unit area than either the CAM or the amnion. Virus production was more apparent in cells between the sinusoids than in those lining them. Intravascular round and filamentous virus was abundant. Virus production in the spleen was similar to that in the CAM and amnion except that the fibrillar or tubular intracytoplasmic inclusions were seldom found (Fig 9).

#### Virus-like Forms

Two locally obtained embryos used for CG infection contained a different type of virus-like particle that did not resemble NDV. The tissues from those embryos had more extensive virus-related cellular change than any of the other embryos examined, and also contained

more large, dark, fibrillar, cytoplasmic inclusions. The virus-like particles had a dense osmiophilic granular core surrounded by one or more concentric layers of granular material of variable electron density. None had an external unit membrane; their outlines were fuzzy. These virus-like forms were found only in the mesodermal layer of these two embryos, generally near the allantoic basement membrane. Unusual forms, resembling organisms dividing by fission, were present in several areas. None was found within cells and replication of these particles from cell membranes was not observed.

#### Chorioallantoic Fluid

Negative stains of the resuspended chorioallantoic fluid pellets contained pleomorphic round or oval virions and occasional fragments or rods of tubular nucleocapsid (Fig 10). Thin sections of pellets contained a wide variety of round and ovoid forms in a heterogeneous matrix of membrane-bound vesicles, distorted mitochrondrial fragments, round, granular electron-dense aggregates unbounded by membranes and occasional clumps of nucleated erythrocytes. In contrast to the findings in embryonic tissue, elongated viral forms were much less numerous in the pellet. None was typically filamentous or as long as those found in the tissues. Most of the viral particles resembled the dark, round and ovoid forms of NDV with randomly coiled nucleocapsid that had been noted in tissues. They ranged in greatest diameter from 1200 to 3600 Å. Many had unit membrane envelopes with external spikes. The smaller particles contained either tightly packed, electron-dense nucleocapsid ribbons, or their ribbons were arrayed in a loose random fashion. Occasional large viral forms had loosely coiled nucleocapsid and no external spikes on the envelopes.

Several pellets, including one from the seed pool ($CG_{13}$), contained unusual virus-like forms different from those of NDV. One type (Fig 11) had the dense granular osmiophilic core and concentric outer layers of variable electron density comparable to those described in tissues. The other type had a unit membrane but was smaller, had no external spikes and its central region was indistinct.

### Discussion

It is apparent from this study that various forms of developing NDV are distinguishable from the normal ultrastructural components of cells in the chick embryo. These cells also have a wide range of normal anatomic variation that is not related to infection or to inflammation. The additional fixation with uranyl acetate enhanced certain features of cell

membranes and of cytoplasmic and microvillous fibrils not previously described in the CAM. The normal features discussed below are not considered virus material or virus-related changes because they were found in inoculated and uninoculated control embryos from local and pathogen-free, CoFAL-negative flocks as well as in NDV-infected embryos.

The cytoplasmic fibrils, 70–90 Å in diameter, and typical microtubules were frequent both in normal and NDV-infected epithelium and in mesodermal fibroblasts. Usually scattered in the cytoplasm, both structures may be mistaken for fibrillar or tubular cytoplasmic nucleocapsid when cut in cross-section or if closely aggregated. However, they are distinguishable from nucleocapsid by their different sizes and typical structure.

Microvilli of allantoic and chorionic epithelium required careful anatomic differentiation from filamentous virus forms, especially at low magnifications, because at times they had relatively comparable diameters and lengths, depending on the plane of section. Microvilli contained slender straight cytoplasmic fibrils, 70–90 Å wide, that lay parallel to the long axis and near the center of the microvillus. In contrast, filamentous virus had peripherally arrayed nucleocapsid immediately beneath the envelope rather than in its center. Depending on the plane of section, the nucleocapsid appeared as ribbons or as round, occasionally hollow dots, 110–150 Å wide. The filamentous forms of NDV found in this study, as well as those of the B1 vaccine strain first illustrated by Bang [6,7,10] and Feller *et al*,[9] have a relatively characteristic appearance. However, the elongated structures with slender central fibrils that Blough [11] considered to be filamentous virus were probably microvilli.

Membrane-bound granular bodies and vesicles in cells of both normal and infected CAM can also be mistaken for intracellular virus particles. Several intracytoplasmic structures designated as viral particles by Bang,[6] and other particles labeled mature and immature virus particles by Blough,[11] are difficult to evaluate. Some resemble pinocytotic vesicles while others are similar to the variably electron-dense granular bodies found in normal [19] and NDV-infected allantoic epithelium. No round or filamentous virus forms of NDV were found within the cytoplasm of cells in the present study, an important feature when considering cell-associated NDV.

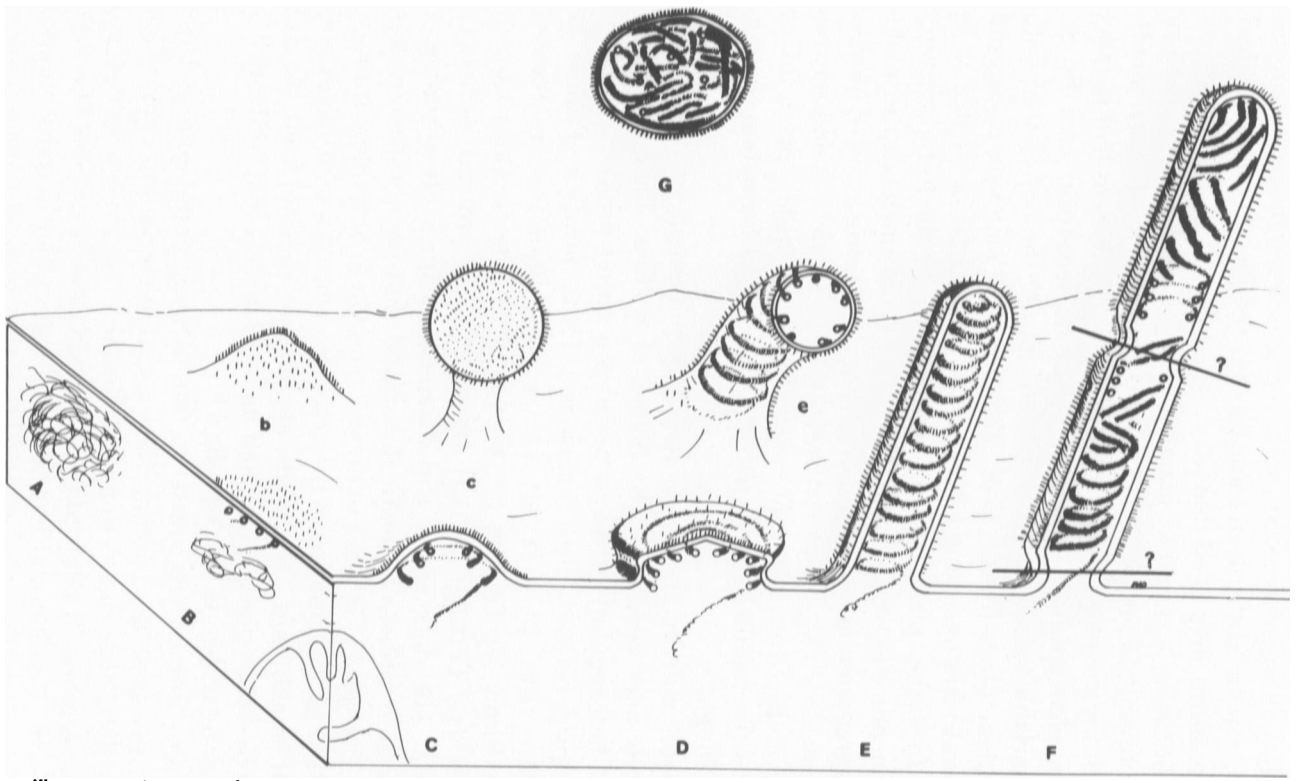
Many different types of vesicles and blebs distorted the tips of microvilli in normal and infected CAM. In this study, comparable structures were also found in thin sections of centrifuged pellets of infected chorioallantoic fluid. Murphy and Bang [20] noted such structures in chromium-

shadowed preparations of centrifuged crude allantoic fluid from both normal and influenza-infected chick embryos. It is probable that these represent a normal process of shedding by the allantoic epithelium, as suggested by Morgan (cited in *Discussion,* Blough [11]).

Replication of the CG strain of NDV used in this study did not involve budding from the tips of modified allantoic or chorionic microvilli in the manner suggested for the chicken avirulent (B) or vaccine (Bl) strains of NDV.[6] Also, the budding process is not like that of murine mammary tumor virus (MTV).[21] Although NDV, influenza A and C, and MTV have relatively similar external appearances,[22–24] MTV appears to have a spherical rather than helical internal component [25] and its morphogenic pattern differs distinctly from that of NDV and the WSN strain of Influenza Ao.[26]

The morphogenesis of NDV in the CAM is comparable to that of other paramyxoviruses grown in cell cultures: SV-5,[27] Sendai,[28] human parainfluenza, type II,[29,30] mumps,[31] WB [32] and measles.[33,34] The budding process appears to result from an undefined interaction between the developing nucleocapsid and a cell membrane rendered capable of forming a virus envelope. The basic patterns observed in our study were similar, regardless of whether the buds and particles were round or filamentous. Text-fig 1 is a semidiagrammatic representation of changes observed in the CAM, amnion and spleen of embryos infected with the CG strain of NDV. The sequence depicted is based on examination of multiple sections from repeated studies. It should not be considered complete, but represents an attempt to arrange the observations of this study in a reasonable order.

Within epithelial and mesodermal cells, the budding process may be preceded by the appearance of dark cytoplasmic inclusions of randomly oriented fibrils, 50–70 Å in diameter (A, Text-fig 1). Cell membranes of the cells containing such inclusions were often dark and thick in focal areas. The subjacent cytoplasm at these sites was dark and fibrillar. When distinguishable nucleocapsid ribbons or dots 110–150 Å wide lay close to these sites, the external surface of the unit membrane had a fuzzy appearance (B, Text-fig 1). Aggregates of tubular nucleocapsid elsewhere in the cytoplasm were barely distinguishable and infrequently found. The buds had a variety of round and flat shapes, some of which probably depended on the contour of available cell membrane. Regardless of the shape of the bud the nucleocapsid ribbon or dots maintained a regularly ordered array beneath the unit membrane (b, c and d, Text-fig 1). Their appearance depended on the plane of section through the buds and the position of the bud relative to the cell membrane.




TEXT-FIG. 1.—Semidiagrammatic representation of possible sequences of morphogenesis of NDV. Fibrillar cytoplasmic inclusion (*A*) may be early stage of nucleocapsid aggregation that precedes formation of tubular nucleocapsid beneath cell membrane (*B,C,D,E*). Spikes on external aspect of cell membrane are usually associated with demonstrable tubular nucleocapsid beneath cell membrane. As nucleocapsid coils, spiked cell membrane bulges (*b,C,D,E*) and either forms round, flat or filamentous forms (*c,D,E,F*). Serial (*E*), transverse (*e*) and tangential (*F*) sections of filamentous, round and other budding forms suggest that nucleocapsid is arrayed in regular helical pattern near attachment of particles to cell. Also, more distal portions (*F*) and free dark round particles (*G*) have randomly coiled nucleocapsid. Constrictions in basal portions of particles near cell of origin or along the lengths of filamentous forms may be part of budding mechanism that results in production of one type of free particle (*G*).

The shape of filamentous virus apparently did not depend on the contour of the cell membrane. Multiple sections through many samples of the CAM, amnion and spleen indicate that the filamentous virus had a relatively uniform shape and diameter.

In many instances a round or filamentous bud had been sectioned in a plane that revealed regularly spaced round nucleocapsid dots immediately beneath the envelope in one area while adjacent areas had parallel ribbons of nucleocapsid. This suggests that nucleocapsid may be arrayed in a variable spiral or helical pattern during the budding of both round and filamentous forms (C–F, Text-fig 1). A single nucleocapsid ribbon, several ribbons or one ribbon drawn out from the center of its length may form the spiral. It is not certain whether the initial changes in the cell membrane require only the presence of nucleocapsid material or its aggregation into tubular form. The symmetric array of nucleocapsid in early budding forms contrasts with its random coiled pattern in more developed forms. In no case did nucleocapsid appear to be laid down in the parallel folded pattern during bud formation, as suggested by Blough.[11]

The variety of light and dark, round and filamentous forms lying between cells may be due to the planes of section. The light round forms often had regularly arrayed peripheral round profiles or ribbons of nucleocapsid beneath their envelopes. These forms and those labeled "mature virus" by Feller *et al*[9] may be oblique sections through filamentous forms (e, Text-fig 1). They may also be sections through round buds (c, Text-fig 1) that have helically arrayed peripheral nucleocapsid. The dark round and filamentous forms often contained randomly coiled ribbons beneath the spiked unit-membrane envelope (F and G, Text-fig 1). It was not always possible to determine if any of the forms that lay separate from the cells were actually continuous with nearby cell membranes. The buds appear to separate from the cell membrane by being pinched off from its basal attachment to the cell or elsewhere along its length (F, Text-fig 1).

Filamentous forms were sparse on the allantoic epithelium and rare in both negatively stained preparations and thin sections of chorioallantoic fluid pellets. Also, the sections of pellets did not contain light, round forms with peripherally arrayed nucleocapsid dots or ribbons like those observed in the mesodermal layers. This suggests that serial sections of such round forms in tissues may reveal that they were oblique or tangential sections of budding virus particles. On the other hand, most of the round forms with variably electron-dense bodies present in the pellets contained either loose or tight random coils of nucleocapsid. The latter resemble virions illustrated in studies of the entry of influenza virus[35]

and Sendai virus [36] into cells of CAM. Hence, the dark forms more likely represent the fully formed virions. Some of the variation in nucleocapsid content, manifested by the looseness of the coiling may be real or it may be an artifact resulting from centrifugation.

There is no ready explanation for the production of filamentous forms by myxoviruses. Most demonstrations have been made in tissue culture systems. Previous reports of filamentous forms of NDV in a system *in vivo* concerned the chicken avirulent [6,7] and/or the Bl vaccine strains,[6,7,9] and the filamentous virus was noted to be confined to the luminal surface of the allantoic layer. Bang suggested that the capacity to produce filamentous virus might be greater in epithelial cells than in fibroblasts and that certain strains of virus produce filamentous forms more frequently than others.[6] The results of the present study suggest that both of Bang's hypotheses are only partly true with respect to NDV because filamentous virus formation is not necessarily the province of epithelial cells or of the chicken avirulent or Bl strains.

In general, the production of filamentous forms or of large cytoplasmic aggregates of nucleocapsid by paramyxoviruses may be due to a limitation of cell division or of cytoplasmic membrane production imposed on the cell by the degree of infection or the type of virus. It may also be due in part to the type of host cell infected. Evidence for each of these possibilities has been provided in studies of SV-5 virus infection of BHF-21F and primary monkey kidney cell cultures [27] and with other paramyxovirus infections in non-natural host systems. SV-5 virus produced numerous filamentous forms in both types of culture. But few cytoplasmic nucleocapsid aggregates were found in monkey kidney, its natural host, while many aggregates occurred in BHK-21F cells. Monkey kidney cells continued to produce large amounts of infective SV-5 virus but the BHK-21F system produced few infective particles.[37] Measles virus infection of primary monkey kidney and BSC-1 cells [34] and of hamster cerebellum cell cultures [38] resulted in large cytoplasmic aggregates of tubular nucleocapsid. The same was true of chick embryo cell cultures infected by mumps virus.[31] Tissue culture studies to evaluate comparable reactions with NDV are currently in progress.

In the same natural host system (the chick embryo) the CG and Bl strains of NDV produce certain similar effects but at different locations, to different degrees, and apparently at different times in the cycle of infection. The Bl strain is reported to produce hyperplasia and hypertrophy of allantoic epithelium [6,9] while the CG strain does not. The Bl strain caused production of filamentous virus on the thickened allantoic layer after 72 hours of infection while the CG strain caused little demon-

strable virus formation of any type in CAM, amnion or spleen before 32 hours had elapsed after infection. However, extensive round and filamentous virus formation was evident in the mesodermal layer after 36 hours of infection. Both strains may produce cytoplasmic nucleocapsid aggregates in the layers where virus formation is most extensive. However, such aggregates were not frequent in this study. The differences in the site of replication and duration of infection prior to demonstrability of virus formation with the electron microscope are quite interesting because Liu and Bang [39] reported that both strains had comparable rates of multiplication in the chick embryo and reached similar maximum titers in the chorioallantoic fluid 24–30 hours after infection.

The vascular involvement found in this study confirms Bang's suggestion [6] that the virulent strain may spread via the blood stream. The ultrastructural demonstration of NDV replication in the spleen of the chick embryo has not been reported previously.

In both the CAM and the amnion, the CG strain of NDV produced large fibrillar cytoplasmic inclusions. Similar, but smaller, fibrillar regions often lay beneath focally darkened cytoplasmic membranes at sites of extensive virus bud production. This change may be the earliest demonstrable aggregation of nucleocapsid. Tubular nucleocapsid was not found within such inclusions as has been noted in tissue cultures of BSC-1 cells [34] and hamster cerebellum [38] infected with measles virus. In the present study, the fibrillar inclusions were more frequent in the two embryos with the most evidence of virus production. Also, they were the only embryos containing the unusual virus-like forms. Although no evidence of their reproduction from cells was observed, the larger virus-like forms may be indigenous to the local flock and may have altered the pattern of NDV replication in affected experimental embryos. The smaller forms resembled those reported by Cromak.[40] They may also be curled fragments of membrane.

In negative stains, care should be exercised in the interpretation of images as myxovirus particles or as nucleocapsid. Sjostrand [41] has shown that unfixed mitochondrial membrane fragments bear knob-like external extensions in negative stains. Because such forms might be mistaken for viral envelopes with external spikes, only those forms containing nucleocapsid strands should be considered virions (Fig 10B). Also, Glauert and Lucy [42] have demonstrated that aqueous dispersions of lecithin, cholesterol and saponin in phosphotungstic acid may form helical complexes and ring structures which, except for their larger size (200–220 Å), might be mistaken for myxovirus nucleocapsid.

## Summary

This ultrastructural study of normal chick embryo chorioallantoic membrane (CAM), amnion and spleen and of similar tissue infected with Newcastle disease virus (NDV) was undertaken to provide a better definition of the morphogenesis of NDV replication in a permissive system *in vivo*. Emphasis was placed on distinguishing virus forms, virus components and intracellular virus-related changes from the wide range of normal ultrastructural features in cells of those tissues.

The CG strain did not bud from the tips of epithelial microvilli in the manner of mouse mammary tumor viruses as previously suggested. Round and filamentous forms developed at the cytoplasmic membrane of cells in all layers of the CAM amnion and spleen by a budding process similar to that of other paramyxoviruses. No round or filamentous forms were found in cell cytoplasm. Light and dark round virus particles varied in the amount and array of nucleocapsid. Sections of pellets from the chorioallantoic fluid contained numerous dark round virus particles similar to those found in tissues, but no light round particles. This suggests that dark particles probably represent late stages in the development of the virus. A possible sequence of morphogenesis is illustrated.

The results of this study are compared with previous reports concerning chicken avirulent and vaccine (Bl) strains of NDV and other paramyxoviruses.

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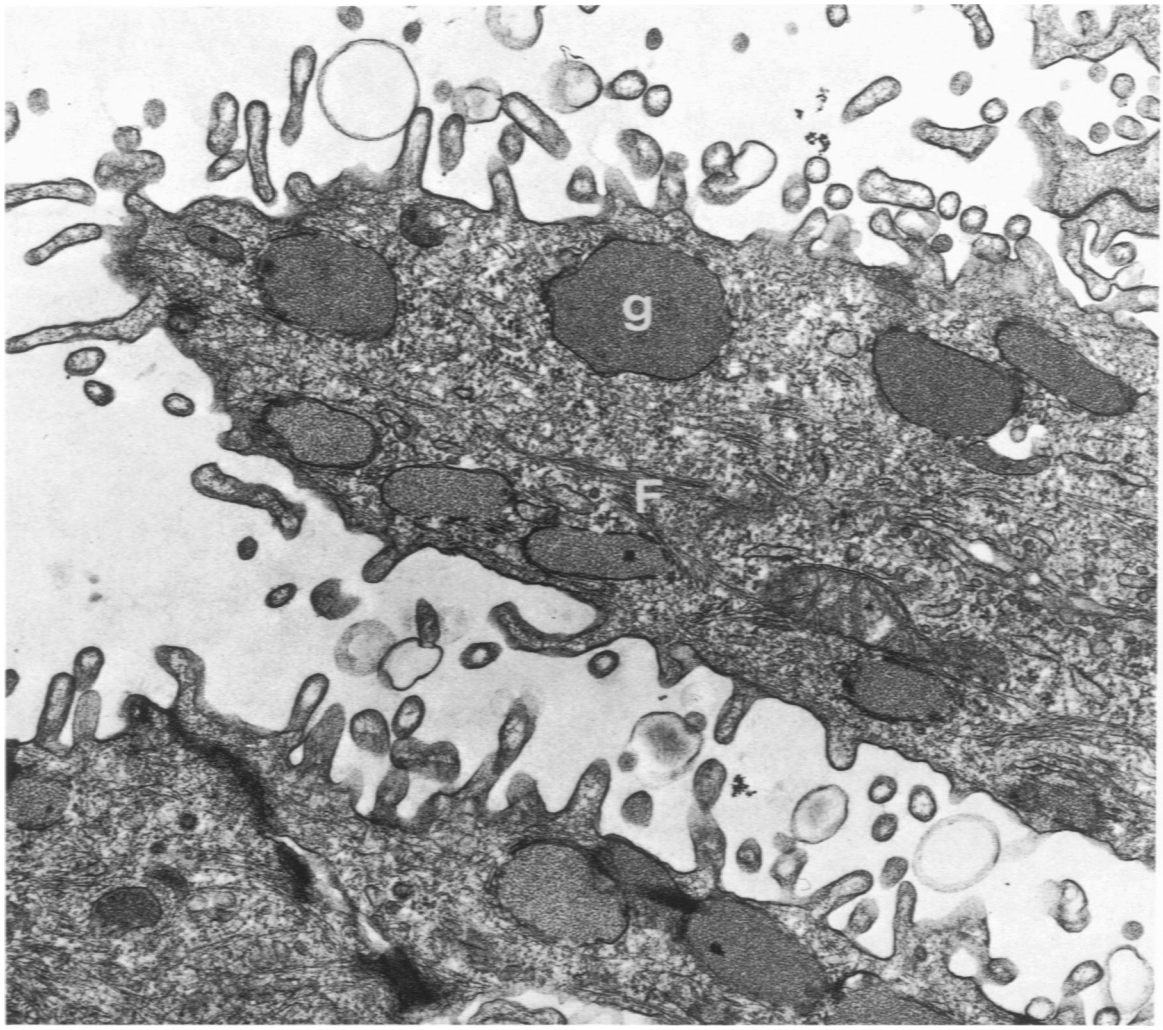
**Fig 1.**—Club-shaped and balloon-tipped allantoic microvilli from 11-day-old control embryo. Cytoplasmic fibrils *(F)*, membrane-bound granular bodies *(g)*. Glutaraldehyde, osmium tetroxide and uranyl acetate fixation. × 17,000.

**Fig 2.**—Cytoplasmic fibrils within allantoic microvilli of 13-day-old control embryo. **A.** Transverse section. × 95,300. **B.** Longitudinal section. × 63,700. Glutaraldehyde, osmium tetroxide and uranyl acetate fixation.

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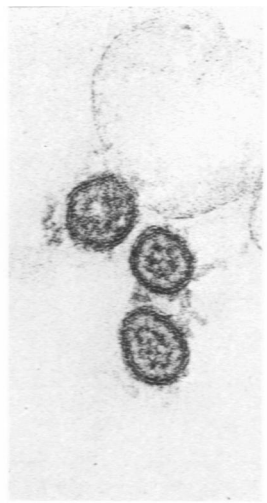

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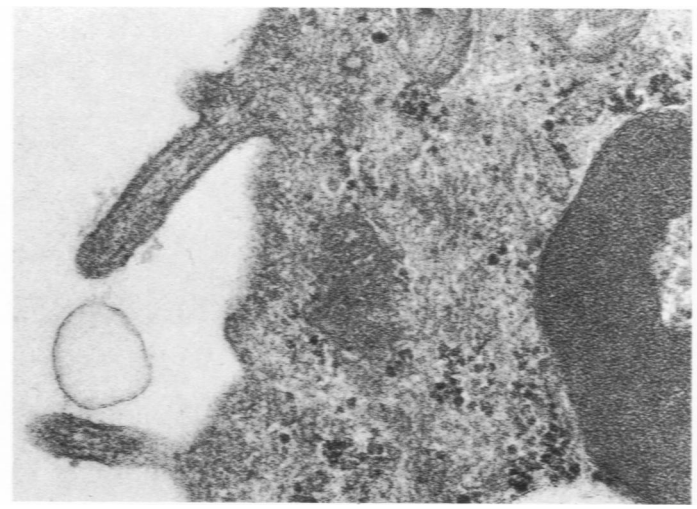

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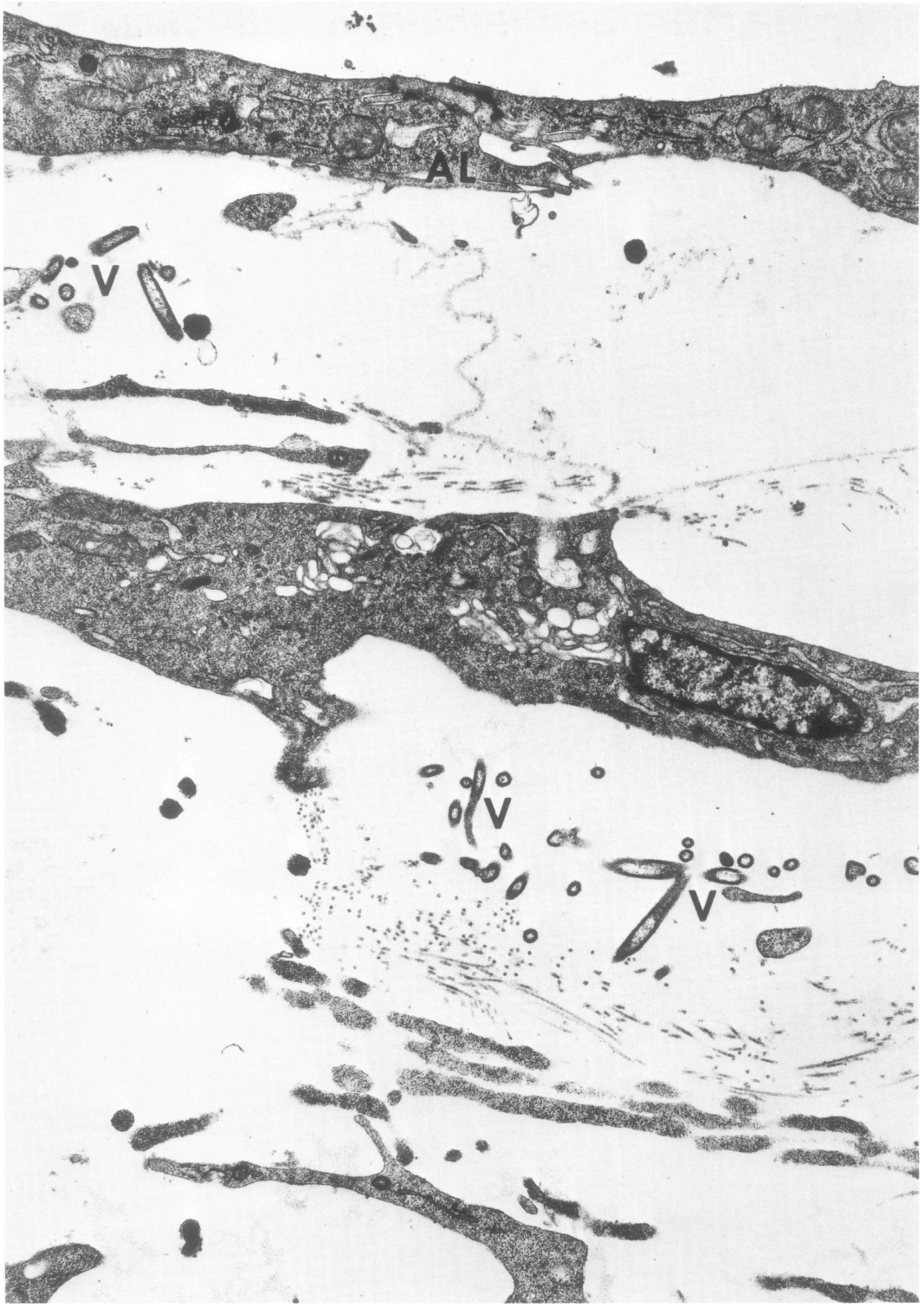
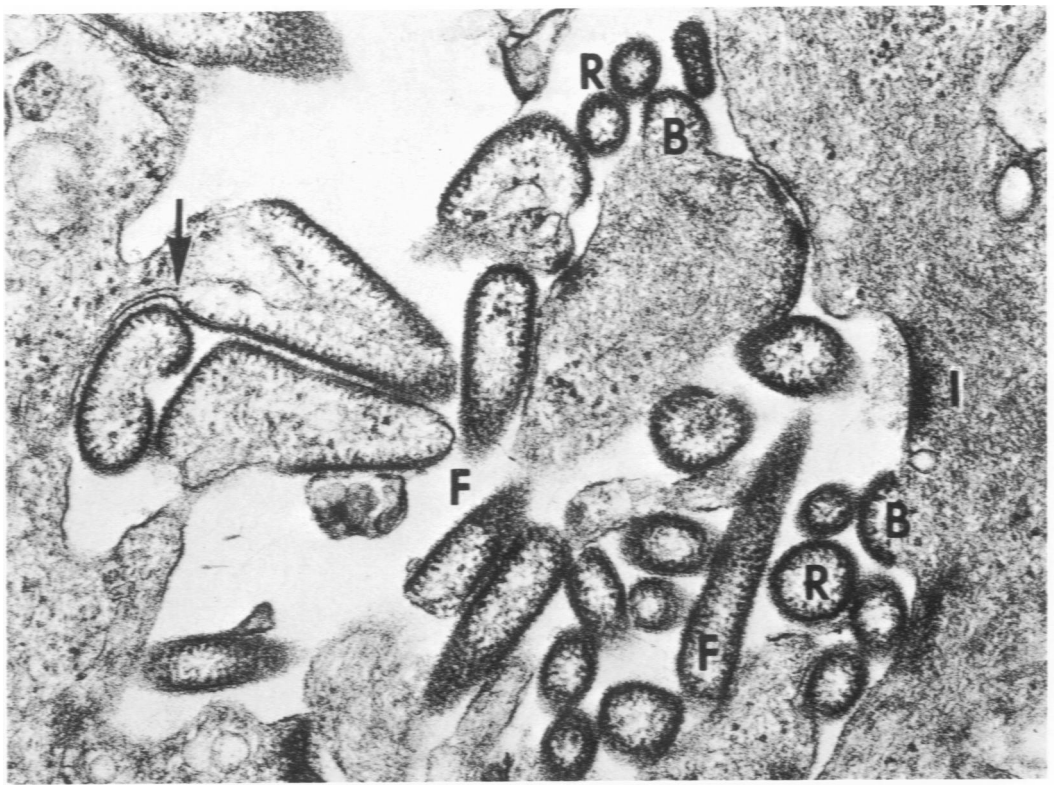



**Fig 3.**—Round and filamentous forms of NDV *(V)* virus below allantoic *(AL)* layer of CAM near mesodermal fibroblast *(M)* after 44 hours of infection. Glutaraldehyde, osmium tetroxide, uranyl acetate fixation. × 13,000.

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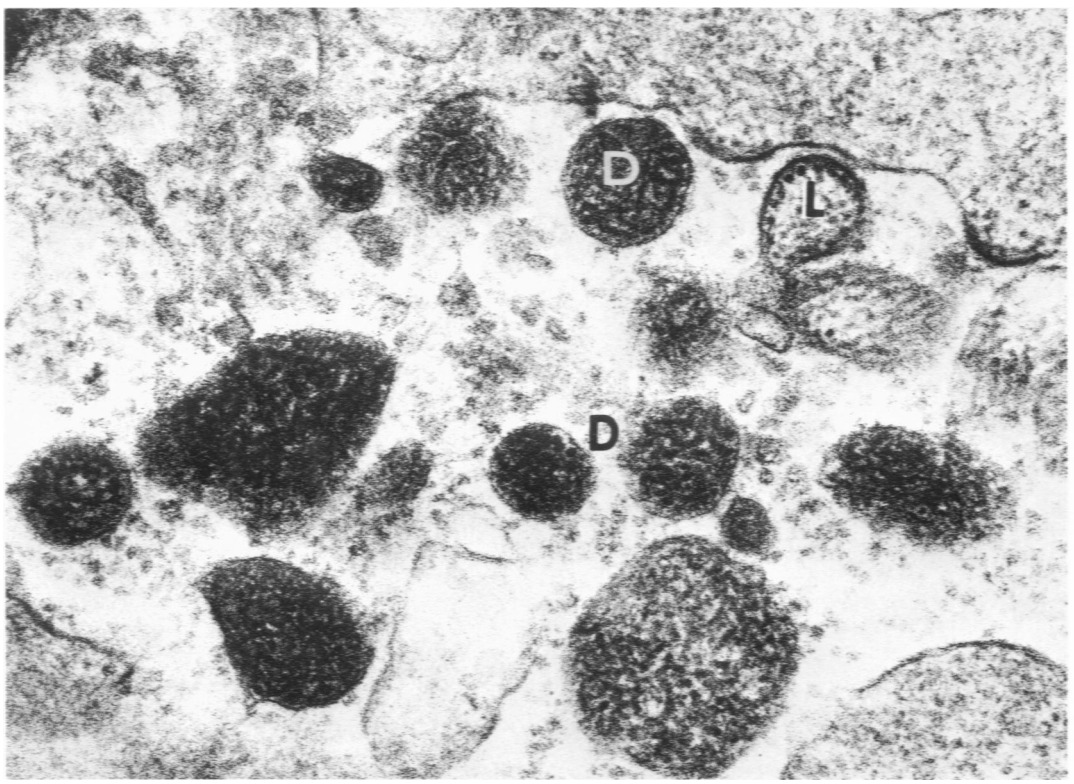


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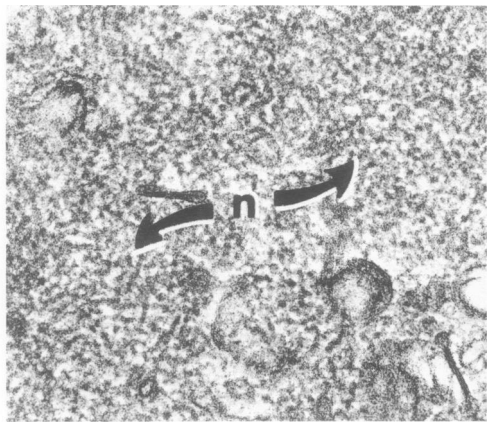
**Fig 4.**—Light round *(R)* and filamentous *(F)* forms of NDV near adjacent cells with surface buds *(B)*. Note distinct separation of inner layers of budding unit membrane *(arrow)* and darkening of cytoplasm *(I)* near budding form. Glutaraldehyde and osmium tetroxide. × 61,600. **Fig 5.**—Light *(L)* and dark round *(D)* virus forms between two cells in allantoic layer. Glutaraldehyde, osmium tetroxide and uranyl acetate fixation. × 92,500.

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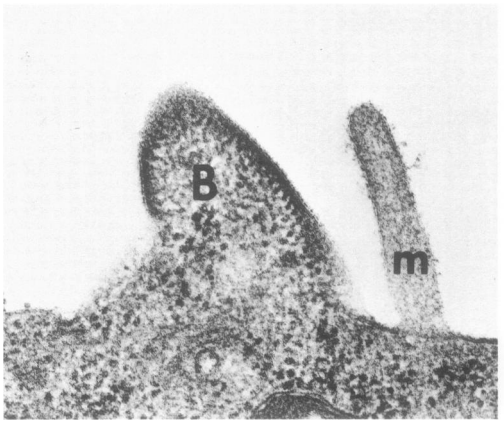


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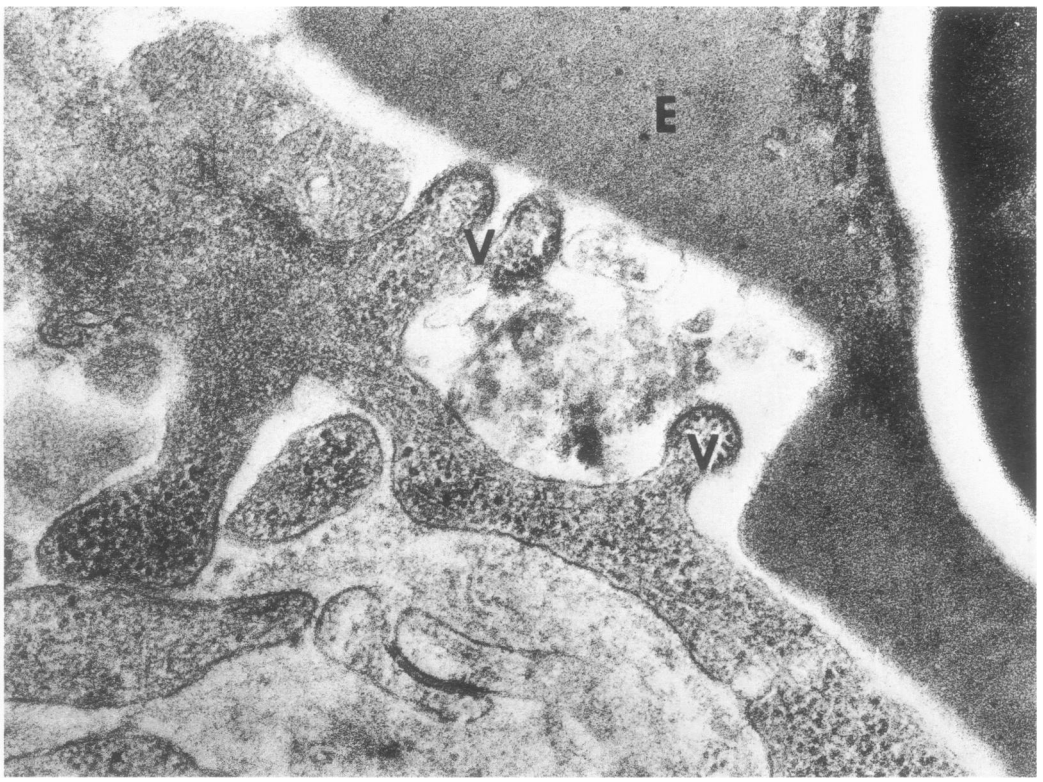


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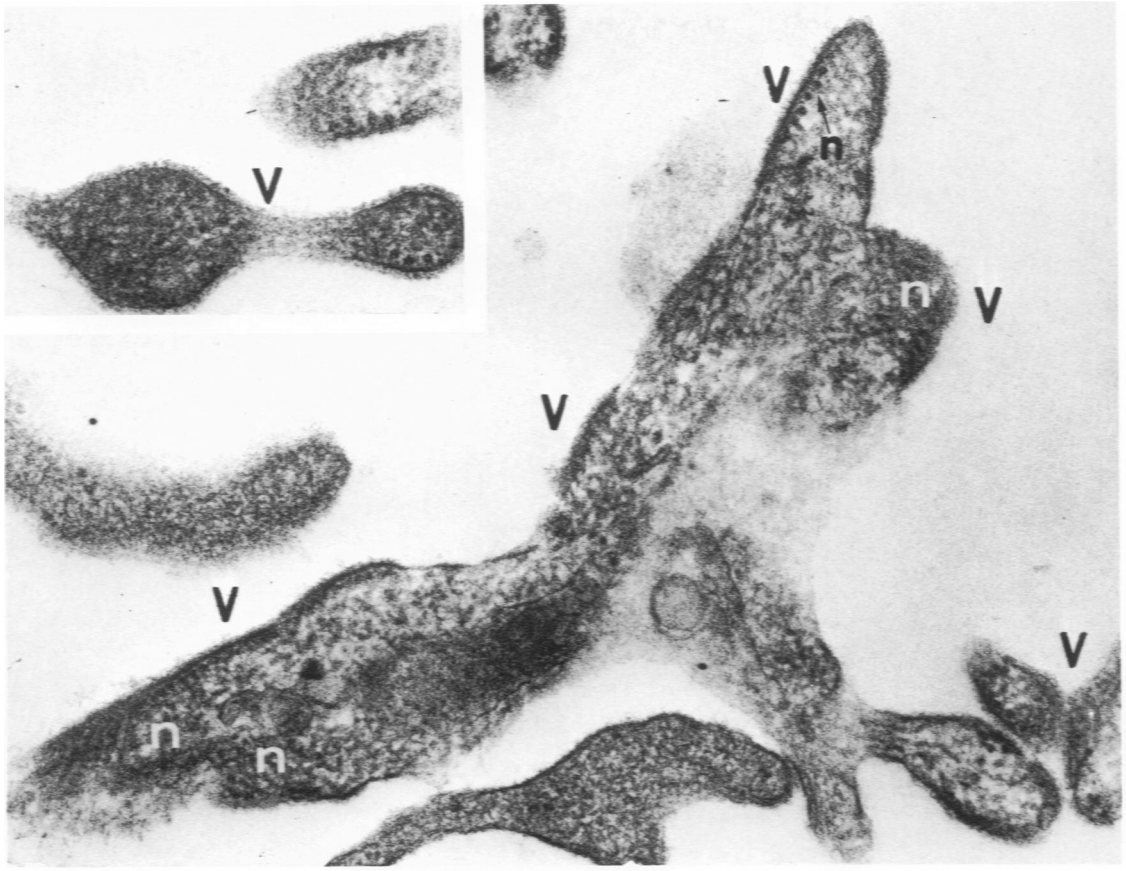
**Fig 6.**—An aggregate of randomly coiled tubular material *(N)* resembling nucleocapsid in cytoplasm of mesodermal fibroblast in CAM. Glutaraldehyde and osmium tetroxide fixation. × 73,500.

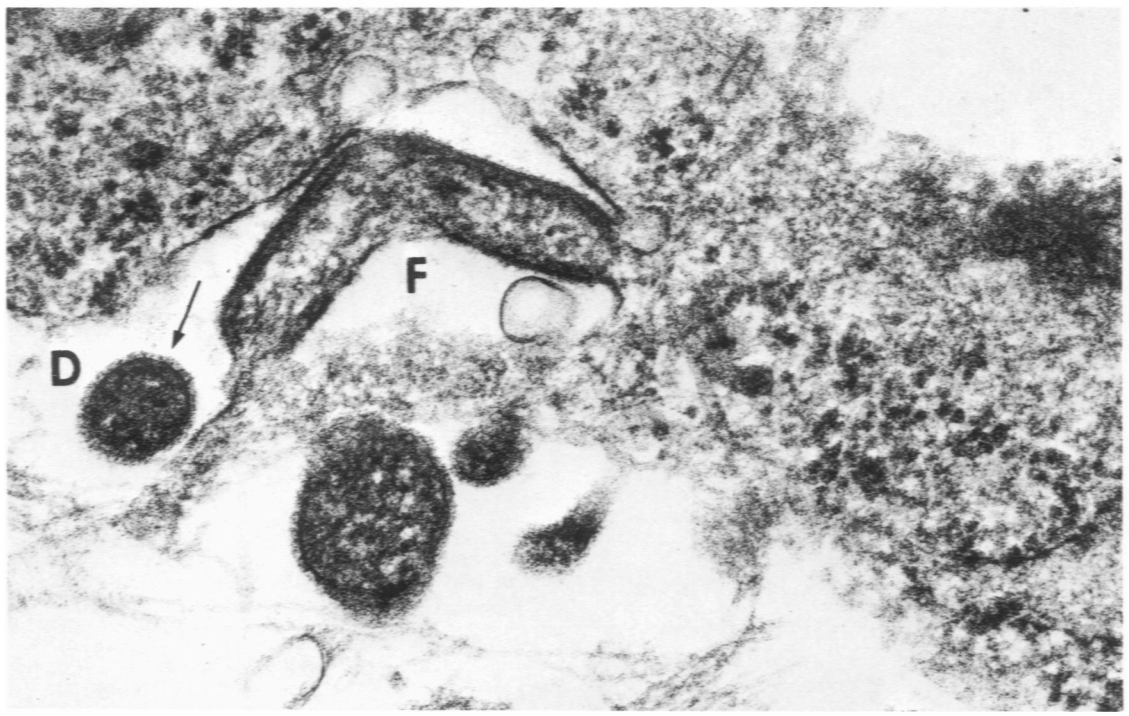
**Fig 7.**—Budding NDV *(B)* near microvillus *(M)* of allantoic epithelial cell. Glutaraldehyde and osmium tetroxide fixation. × 54,200.

**Fig. 8.**—Vascular invasion by budding forms of NDV *(V)* in mesodermal vessel. Erythrocyte *(E)*. Glutaraldehyde, osmium tetroxide and uranyl acetate fixation. × 111,000.

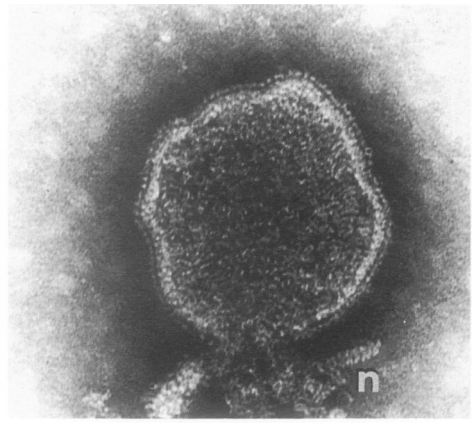
**Fig 9.**—NDV replicating in chick embryo spleen. **A.** Budding forms of NDV *(V)* in splenic fibroblast. Note aggregated strands of tubular nucleocapsid *(n)* in cytoplasm beneath darkened unit membrane and regular pattern of nucleocapsid in cross-section *(arrow)*. Glutaraldehyde and osmium tetroxide fixation. × 72,800. **Inset.** Dumbbell form of NDV *(V)* with fuzzy external layer and nucleocapsid cross-sections beneath envelope. Adjacent form is probably tangential section of filamentous virus. Glutaraldehyde and osmium tetroxide fixation. × 90,000. **B.** Filamentous *(F)* and dense round forms *(D)* of NDV in splenic fibroblast. Note fuzzy external layer of spikes *(arrow)* on envelope of dense virus particle. Glutaraldehyde and osmium tetroxide fixation. × 117,000.




9A




9B

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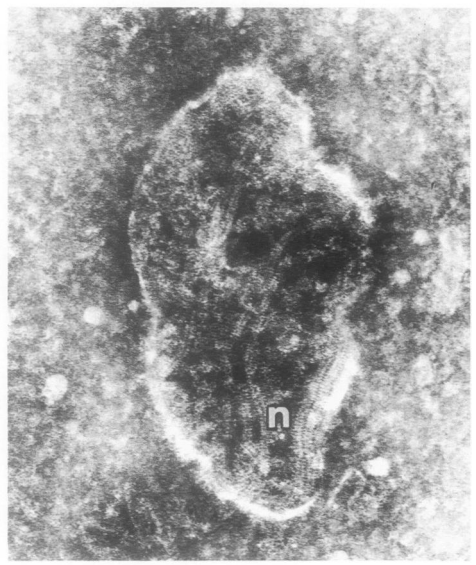


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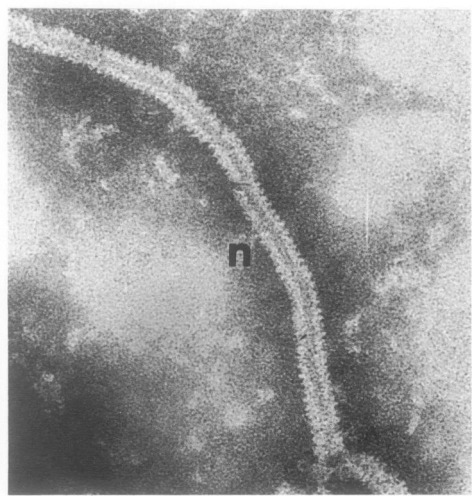


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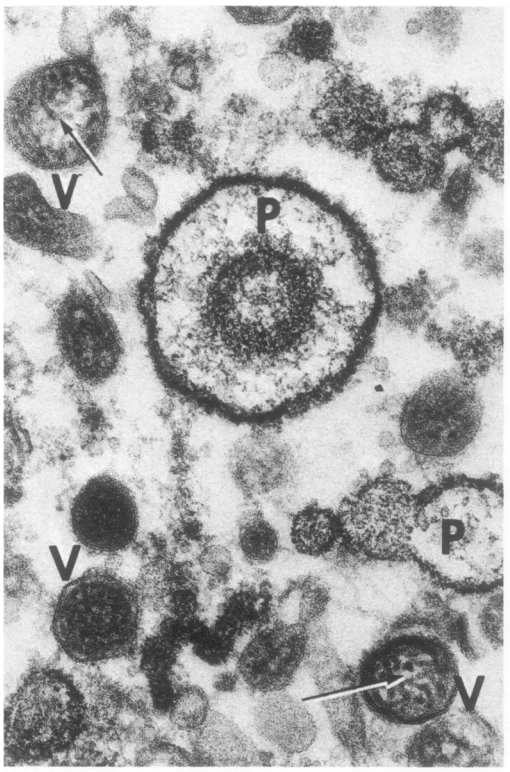


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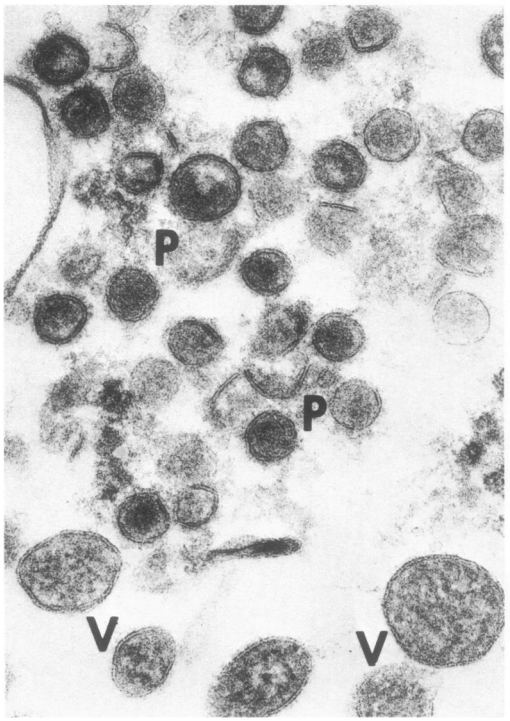


11B

**Fig 10.**—Negatively stained NDV from centrifuged chorioallantoic fluid. **A.** Round form with external spikes. Nucleocapsid *(n)* is apparent near partly disrupted envelope. Phosphotungstic acid (PTA). × 163,800. **B.** Oval virion with internal staining of nucleocapsid *(n)*. PTA. × 92,000. **C.** Nucleocapsid fragment *(n)*. PTA. × 242,000. **Fig 11.**—NDV *(V)* and unidentified virus-like particles *(P)* in sections of chorioallantoic fluid pellets. **A.** Note randomly coiled nucleocapsid strands *(arrow)* and unit-membrane envelope in NDV *(V)*. Virus-like particles *(P)* generally had dense centers surrounded by less electron-dense layer and dark outer ring. Glutaraldehyde and osmium tetroxide fixation. × 62,500. **B.** Virus-like particles *(P)* have surrounding unit membranes but are smaller than NDV *(V)* and may be blebs from microvilli or curled membrane fragments. Glutaraldehyde and osmium tetroxide fixation. × 84,000.