OBSERVATIONS WITH THE ELECTRON MICROSCOPE ON CELLS OF THE CHICK CHORIO-ALLANTOIC MEMBRANE INFECTED WITH INFLUENZA VIRUS*

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PLATES 13 TO 18

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Mosley and Wyckoff in 1946 (1) first called attention to the occurrence of filaments associated with influenza virus in preparations of allantoic fluid from infected chick embryos. In three different strains they showed that these filaments had the same diameter as the spherical virus and that the filaments were occasionally segmented into virus-sized units. Subsequently it was shown (3–5) that the filaments were present in a number of other strains and that they could be adsorbed (2, 3) and eluted (5) from red cells. Recently isolated strains show a greater prevalence of filaments (5), but they have been found in all strains of influenza examined (4), including the recently designated influenza C (6).

A preliminary study of tissue cultures infected with the virus of PR8 (influenza A) revealed spherical virus particles and filaments occurring in the same areas. The filaments were sometimes segmenting and occasionally appeared to be disintegrating and releasing spheres (4). Observations of similar segmented filaments in tissue cultures of fowl plague have recently been reported by Flewett and Challis (7).

The relationship of the spherical virus, the filaments, and their hosts cells has now been further studied with the electron microscope in both tissue culture and chick embryo infections. It is evident that filaments and spherical virus particles project from the infected cell surface and that these forms may be present at the cell surface as late as 48 hours after infection without other cytological change. The nature and variation of the normal cell edge is also described in this paper.

The results of the three different methods of preparation of infected cells

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for study with the electron microscope will be taken up separately. The first method is the Maximow flying coverslip tissue culture (8, 9) of preinfected chicken chorio-allantoic membrane. The second utilizes roller tube cultures (10) infected after 48 hours *in vitro*, and the third consists of making sections (11, 12) of chick membranes infected 24 or 48 hours previously.

MATERIALS, METHODS, AND FINDINGS

Virus.—Three strains have been used in our tissue culture studies. Influenza A (PR8)[1] (13), influenza A prime (FM1)[2] (14), and swine infleunza V15[1] (15). All were prepared from allantoic fluid passaged in 10 to 11 day old embryos. Virus was harvested after 40 hours incubation at 35°C. Stock virus was put up in 1 cc. aliquots in lusteroid tubes, and kept in a dry-ice deep freeze. Allantoic fluid was prepared for electron microscopic observation by fixing ultracentrifuged concentrate with osmium tetroxide vapor. Infectivity titrations were made by allantoic inoculation of serial tenfold dilutions of virus in 0.01 M phosphate-buffered saline. After 40 hours of incubation at 35°C., allantoic fluid was harvested and tested for chick cell hemagglutinins.

Findings in Maximow Coverslip Preparations

This type of tissue culture of preinfected allantoic membrane was the simplest method by which to observe cells 40 or more hours after infection and it yielded a high ratio of infected cells.

Method.—Seven or 8 day old chick embryos were inoculated on the dropped membrane with 0.2 cc. of a 1–10 dilution of allantoic fluid (10^9 infective units of influenza virus per cc.), dilution being made in 0.01 M phosphate-buffered physiological saline. Strains used in this experiment included PR8, FM1, and swine influenza. The embryos were incubated at 35°C. for 24 to 48 hours and the chorio-allantoic membranes removed and placed in small individual Petri dishes containing 1 cc. either Earle's solution (16) buffered at pH 7.4 or Simms's Z solution (18). As soon as possible, an untraumatized area of about 1 sq. cm. was selected, minced finely, and spread on a thin formvar-coated 11 mm. sq. coverslip mounted on a 1 × 1½ inch slide. As much fluid as possible was then removed and two drops of Simms's ultrafiltrate (17) added to the tissue fragments. A Maximow slide was quickly lowered over the coverslip which was sealed in with a thin layer of physiological salt solution. Thereupon the entire assembly was inverted, and after carefully sliding the glass cover aside, 5 per cent CO_2-95 per cent oxygen was blown slowly into the cavity for 10 to 15 seconds and the cover quickly replaced. The slide was then sealed with paraffin and reinverted. When all cultures were finished the Maximow slides were stacked with split rubber tube separators, and placed in a sealed jar with a burning candle inside to increase the CO_2 content. Penicillin was added in some experiments, but, although it produced no recognizable change in the cells, it was discontinued and very few cultures were contaminated.

After incubation at 35°C. for 17 to 24 hours, about one-half of the tissue fragments grew out sheets of very thin epithelial cells. The remainder of the fragments either failed to grow or produced hollow balls. The longer the infection had been present in the embryo before the chorio-allantoic membrane was explanted, the less well the virus-infected cultures grew.

After 24 hours' incubation, the cultures were opened one at a time, carefully washed for 10 to 15 seconds in one of the balanced salt solutions and replaced in their Maximow slides

[1] Courtesy Dr. R. E. Shope.

[2] Courtesy Dr. T. G. Ward.

as a hanging drop preparation over 4 drops of fresh 2 per cent osmium tetroxide. The slides were sealed with salt solution and kept at room temperature in a sealed jar for 5 to 7 hours. The coverslip bearing the culture was then removed and washed first in the balanced salt solution (16, 18) for 5 minutes then in a 50 per cent dilution of this in distilled water for 5 minutes. Finally, it was placed in distilled water and the original bit of tissue was removed. The remaining thin outgrowth on the formvar film was then transferred to wire grids for electron microscopy (9). All preparations were dried overnight over phosphorous pentoxide and lightly coated with chromium (19).

Ninety-four satisfactory cultures were made. One-third of them were infected with influenza, one-third with other viruses, and one-third were uninfected. The majority of the influenza preparations were infected with the PR8 strain of influenza A. To make certain that all preparations were examined with equal care, these experiments were set up by one of us to include in each group (*a*) influenza virus, (*b*) heat-inactivated influenza virus, (*c*) one or another virus from the laboratory stock, and (*d*) a heat-inactivated virus. Following this another person renumbered the preparations at random and kept a code record. The cultures were then studied in the electron microscope without knowledge of the source of the individual specimen. Throughout the study an EMU, 50KV, RCA electron microscope was used. The miscellaneous control viruses included Newcastle (CG) (20), mumps (Enders) (21), vaccinia (22), and fowl pox (23). In all cases infections with influenza virus could be differentiated from the others.

Results.—In a previous report (4), we gave our data on what remained after mechanical removal of the colony. Conclusions as to cytological changes were not justified in these areas but it was shown that the virus particles were present in close association with the influenza filaments in the remnants of the infected culture, and that various gradations between unsegmented rods and chains of spheres strongly suggested a link between the two forms (4). We have extended this work to include preparations of the FM1 strain of influenza A prime (Fig. 1) which produces more long filaments in allantoic fluid, and the V15 strain of swine influenza (Fig. 2) which produces many short filaments and only occasional long ones. These differences were not as marked in the tissue cultures, but swine influenza (V15) did yield a higher proportion of short filaments both on the infected cells and free in the surrounding fluid.

Study of the thin cells which migrated out from the infected explant yielded another type of information. Spherical virus particles and typical influenza filaments were seen sharply outlined on the surface of an occasional isolated cell (Fig. 3). The particles appeared identical with influenza virus from allantoic fluid. The filaments showed parallel sides and greater density than the cytoplasm. The distal ends of the filaments often had a virus-sized nodule of higher density, while the proximal end tended to be squared.

Since the filaments and spheres were elevated above the cell surface, as indicated by the length of the chromium shadow, and since the filaments often extended unchanged across nuclear and cell boundaries, it was assumed that they projected from the cell. This was later confirmed by study of thin sections.

The question of whether the filaments and spheres seen in association with these cells might not have been adsorbed as such on the cells was studied by

placing high concentrations of virus on cultures and fixing. The distribution of adsorbed virus on these cells was uniform. In contrast, in the older infections, several areas were found in which only one cell in a large thin sheet showed any recognizable virus (Fig. 4).

In occasional cultures, evidence of cell degeneration was seen in the light or electron microscope. This was usually due to improper control of the pH, drying of the culture, poor media, or bacterial contamination. A study of these cultures provided data on non-specific changes. Among such changes were a swelling of the mitochondria and an increase in the number of fat droplets and vacuoles. Round sac-like objects appeared, particularly at the cell surface. These were apparently released from the cell as they were also found in the surrounding fluids (Fig. 5). This vesicle formation is very general and has also been seen in degenerating bacteria and fungi. K. R. Porter (24) believes that these vesicles, when present in tissue culture, represent changes due to non-specific injury.

Free floating sacs or blebs were seen in nearly all specimens of allantoic fluid (Fig. 6), whether normal or infected. They appeared as flattened discs with a diameter of 100 mμ to 1 μ and superficially resembled minute human red cell ghosts. These objects can be distinguished from influenza virus by their low density and highly variable size. Although changes of this type were frequently seen in tissue cultures, they were not found as a necessary accompaniment of infection with influenza.

Results in Roller Tube Cultures

The roller tube method (10) was adopted to give data on the first few hours after infection, since it provides a simple technique for producing tissue cultures of good quality with sufficient media to follow virus titrations. It also has been shown to be an effective way in which to culture influenza virus (25).

Method.—New pyrex test tubes were cleaned with duponol C, washed with distilled water, rinsed in 95 per cent alcohol and dried. They were then filled with 0.25 per cent formvar in ethylene dichloride, drained, dried rapidly by water suction, and stoppered with sterile cotton plugs (26). The tubes were used the following day, when tissue cultures of 7 or 8 day old chick membranes were set up in the lower half of the tube in 1 cc. Simms's ultrafiltrate plus 20 per cent fresh cockerel serum. The bottom half of the tubes were first exposed to the media to moisten the formvar membrane and the tissue fragments were then placed on the tube walls. The tubes were then left standing in the incubator for 3 hours before being started in the roller drum. This increased the number of explants which adhered to the tube wall. The tubes were incubated for 48 hours at 35°C. and virus was then introduced. In these experiments, fresh swine influenza virus was added. This had been concentrated 10 × by centrifuge and was added in 0.1 cc. amounts. Incubation in the roller drum was continued for variable lengths of time after infection.

At the end of incubation the tube was reopened, emptied of media, filled with a balanced salt solution (16, 18), and drained immediately. Fixation was accomplished by closing the tube with a rubber stopper into which a precipitin tube had been inserted. This small tube

contained 0.1 cc. of osmium tetroxide solution which vaporized into the roller tube. The osmium fixation was continued for 1 hour. The culture was washed in the same balanced salt solution for 5 minutes, then in a 50 per cent dilution of this in distilled water for 5 minutes, and finally in distilled water. The tube was then broken into pieces. The cells on the membrane were harvested from these glass fragments and transferred to electron microscope grids. The preparations were dried and shadowed as above. Observations were made on 40 tubes inoculated with swine influenza.

Results.—In these experiments concentrated virus was inoculated in an amount sufficient to provide several particles on each cell. Swine influenza was used, since filaments were rare in the inoculum. Therefore, if any number of influenza filaments were found in the tissue culture they had presumably been produced there.

One difficulty was immediately apparent. Some of the cells, whether control or infected, were found to have numerous finger-like projections on their surface. However, these "normal" filaments are different from influenza virus in several ways. They are pleomorphic, most being slightly larger, they do not have as great a density, they have irregular width, they tend to be club-shaped, and they do not show the highly characteristic terminal virus-sized nodule. Fig. 7 shows some of the "normal" filaments, which have the greatest similarity to influenza.

Because of these normal filaments it was not possible to follow virus development in a period less than 5 hours after infection. This difficulty was demonstrated in the following way. All photographs from these preparations were mixed with no labels other than a code number, and classified as to whether virus filaments were present or not. The only preparations which could be positively identified as showing virus filaments were in the group fixed 5 hours or more after inoculation (Fig. 8).

Scattered virus particles were seen evenly distributed over the cells at time zero, and also in cultures fixed 12 hours later. Typical influenza filaments which had apparently come off could sometimes be seen on the substrate after 5 hours of infection. In the 5 to 12 hour preparations, virus in higher concentration than in the inoculum was found on some cells and sometimes at the colony margins. No evidence of cell destruction was seen during 12 hours of infection, although there was morphological evidence of virus growth.

A significant rise in virus titer was not demonstrated in any of these experiments, either by repeated embryo titrations or by cell agglutinations. This is not surprising since there is very little tissue in these cultures, and a small increase in virus would not significantly affect the original titer of 10^9 or 10^{10} virus particles per cc.

Findings after Infection of Chick Embryos

Although the tissue cultures proved to be a useful experimental model, the cells were not in the same environment as in the intact embryo. For this

reason infected allantoic fluids and sections were studied. The results correlated well with the tissue culture observations.

In an effort to find out if filaments or spheres appeared at different times in the embryo the following experiment was done.

Method.—Twenty-four embryos were inoculated intra-allantoically with approximately 10^{+8} (10 per cent allantoic fluid) infective doses of influenza A (PR8) and the allantoic fluids harvested at 4 hour intervals individually. Fluids were tested for ability to agglutinate chicken red cells and a sample of each fluid was spun at 14,000 R.P.M. in a motor driven (27) centrifuge for 45 minutes at 4°C. Most of the supernate was discarded and the remaining drop was used to resuspend the sediment. Electron microscope preparations were made with this material and fixed with 2 per cent osmium tetroxide vapor for 10 to 15 minutes.

Results.—Examination immediately after inoculation, and in several embryos even later, showed only an occasional virus particle. This amount of virus was insufficient to give visible agglutination of red cells. With the production of increasing amounts, *i.e.* 8 to 12 hours after inoculation, virus could be detected both in the electron microscope and by red cell agglutination. However, the ratio of filaments to spheres was similar to that in the 48 hour fluid. Presumably then the filaments and spheres are continuously produced.

Preparation of Sections for Electron Microscopy

It was originally shown by Baker and Pease (11) that sections thin enough for electron microscopy could be prepared with certain modifications of the Spencer rotary microtome. Sections of infected chick embryos (22, 23, 28, 29) have been prepared by another modification (12) and are valuable in the study of virus infections.

Method.—Ten or 11 day old embryos were inoculated either by injecting 0.1 cc. of phosphate buffered saline containing 10^6 virus/cc. into the allantoic fluid, or by placing 0.2 cc. of the above suspension directly on the allantoic membrane. Controls received the same suspension after heating to 56°C. for 30 minutes. After 24 to 48 hours incubation of the embryos at 35°C. the chorio-allantoic membranes were carefully removed, placed in 2 per cent osmium tetroxide solution overnight and washed in tap water. Dehydration with alcohol, embedding with a mixture of methyl and butyl methacrylate, and sectioning were done by the method of Newman, Borysko, and Swerdlow (12). Sections were obtained from chorio-allantoic membranes infected with PR8 and swine influenza including controls inoculated with heat-inactivated virus (56°C. for ½ hour). The sections were prepared by Mr. Fred Ise and Mr. Emil Borysko. Other studies done in this laboratory on sections of allantoic membrane infected with other viruses served as further controls (22, 23).

Results.—The first and most striking finding in both normal and infected membranes was the number of separate and discrete finger-like projections which were found at some cell borders whether fixed with 2 per cent osmium tetroxide (Fig. 9) or with 10 per cent neutral formalin. These blunt projections are limited to the cells lining the allantoic sac and are similar to those de-

scribed in infections of the chorio-allantoic membrane with vaccinia (22), and probably similar to the normal filaments seen in the roller tubes.

It was relatively difficult to find virus in these sections, even though the virus had been inoculated 48 hours before the specimens were obtained. However only a very thin strip of the free cell surface, often no thicker than the virus itself, is contained in the section. This means that a section may be obtained from a moderately heavily infected cell without including more than one or two virus particles or filaments. Therefore, one can expect to identify infection only in cells heavily burdened with virus.

The general observations on virus made in the tissue cultures were confirmed by a study of the sections. Virus particles and filaments were seen in association with each other, projecting from the cell membrane. Some examples of segmented filaments were seen (Fig. 10). No particle identifiable as virus was seen except at the intact cell surface (Fig. 11). The virus particles and filaments can be differentiated from the normal projections here as in the tissue culture by their more uniform width and character, by the presence of terminal nodules, and by the greater length in some cases (Fig. 10). The majority of infected cells appeared entirely normal, save for the presence of virus. A few nuclei contained some thin dense material diffusely distributed (Fig. 10), the origin of which is not known.

DISCUSSION

Three general problems have arisen in the course of this study. (*a*) How can we identify the virus and differentiate it from the normal filaments? (*b*) When and where do the virus filaments and particles appear? (*c*) How is the virus released from the cell?

(*a*) *The Identification of Influenza Virus.*—Identification in allantoic fluid is relatively easy, since the various cell degeneration products have a lower density than the virus, and "normal" filaments have not been seen in any number in allantoic fluid.

However, in tissue cultures and sections the problem is more difficult. Controls in both cases included tissue exposed to heat-killed virus, and a comparison was made with tissue infected with other viruses. We identified as influenza virus only those filaments which were identical in size, shape, and density with those found in allantoic fluid, and as spherical virus particles those with the same size and density as the influenza from the allantoic fluid.

In a preliminary note by Eddy and Wyckoff (29), changes have been described in sections of influenza-infected allantoic membranes and mouse lung which are interpreted by the authors as "developing" influenza virus. They do not mention the "non-viral" filaments and did not find filaments in control preparations.

We have not studied sections of mouse lungs, but in our experience, normal

filaments are by no means present on every cell of the allantoic membranes. Such filamentous processes as may occur on normal cells must be carefully differentiated from possible virus by a comparison of size, shape, and density. The exact relationship of the virus filaments to the "normal" filaments, if any, remains unexplained. The relationship of the virus filament to the spherical virus is also incompletely understood. It is possible that only very short filaments represent optimal virus growth, whereas, long filaments represent a decreased rate of segmentation. All gradations from spherical particles to filaments, a micron or more in length, may be produced, as is the case in most strains.

(*b*) *Distribution and Development of Virus.*—Virus which was distributed evenly on the cells was observed after inoculating tissue cultures in the roller tube with high concentrations of virus. The same approximate concentration and distribution were found in similar cultures throughout the period studied; *i.e.*, up to 12 hours.

At 5 hours and later, occasional cells from the roller tube cultures showed typical influenza filaments projecting from their surfaces and an increase in spherical particles at their surfaces in comparison with nearby cells. No earlier stages in this phenomenon could be differentiated from the normal cell surface projections. The finding of filaments at this time after inoculation is encouraging, however, since Henle (30) demonstrated that swine influenza and influenza A show new virus production after 5 hours in the embryo.

In such cultures, infected after the establishment of a colony of cells, no virus was seen under the colony when this was mechanically removed. This is in contrast with the colonies grown from tissue already infected, in which virus may be found on either the upper or lower surfaces. In sections filaments and spheres have been seen only at the exposed surface.

(*c*) *Method of Release of Virus from the Cell.*—Throughout this study search was made for evidence of cell rupture with release of influenza virus. Furthermore, efforts were made to detect a cytological change which might represent an intracellular virus phase. Within the limitations of the method, which only allows us to identify formed virus particles, no evidence of any of these changes could be found. Early positive findings were limited to the changes in the exposed cell membrane. Changes appeared in the nucleus later. These latter need further study.

In the embryonated egg new virus is produced a few hours after the original infection of cells has taken place (30). If virus is released then without rupture of the cell, there must be some other means of liberation. As has been pointed out earlier, the influenza filament projects from the cell surface and occasionally appears to be composed of beaded, virus-sized units. The separation of a virus particle from the filament would release the virus for infection of another cell.

Eddy and Wyckoff do describe necrosis, vacuolization, and the occurrence

of virus particles "deep within cells" and "developing out of the cytoplasm of disintegrating cellular edges." We have not observed this during the first 48 hours of infection, but such changes might appear later or during infection produced by more destructive strains of virus. However, the important point in this study is that virus has been apparently produced before these pathological changes are manifest.

SUMMARY

Preparations of influenza-infected chick chorio-allantoic membrane made by two types of tissue culture and by sectioning, have been studied in the electron microscope. Comparisons have been made of influenza A′ (FM1), influenza A (PR8), and swine influenza (V15), three strains which produce different relative numbers of filaments.

Normal surface projections which may be confused with influenza filaments are described. Extruded products of degenerating cells, usually bleb-shaped, may also be found both in uninfected allantoic fluid and tissue cultures.

It appears that the filaments and spheres of influenza virus, concurrently projecting from the free cell surface, represent the only visible change in the cells until late in the infection,—how late the present work does not tell. No definite evidence of a generalized infection throughout the cytoplasm or of inclusions was found. Additional evidence is presented for the assumption that the filaments have a significant role in the final development of the free virus.

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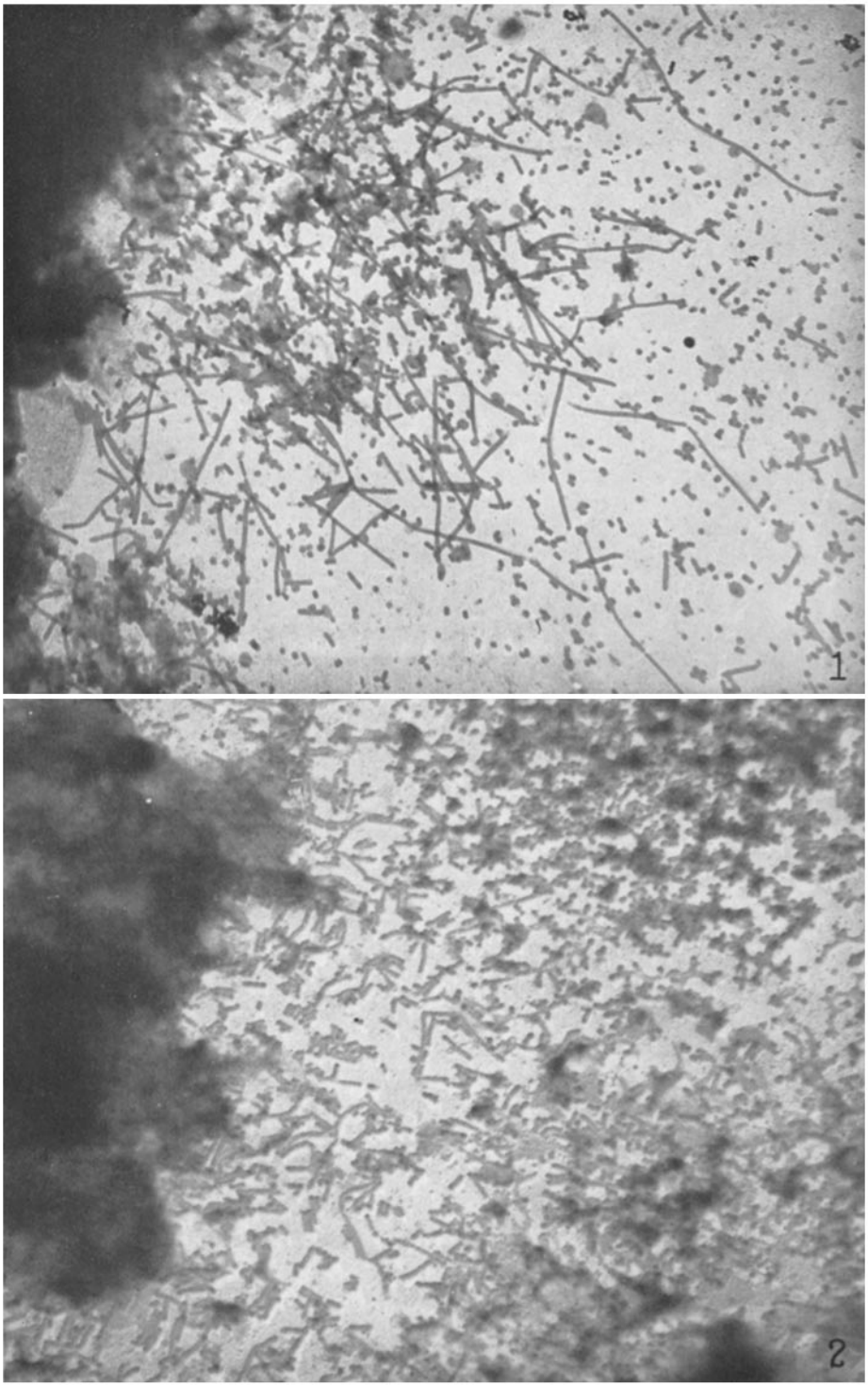
EXPLANATION OF PLATES

PLATE 13

FIG. 1. An area from a Maximow slide tissue culture infected with influenza A prime (FM1) from which the cells have been removed. Long filaments and many spherical particles are seen. Examples of segmented filaments appear in the upper right.

FIG. 2. A similar preparation except that it is infected with swine influenza V15. Many short filaments and spherical particles are seen. × 8600.

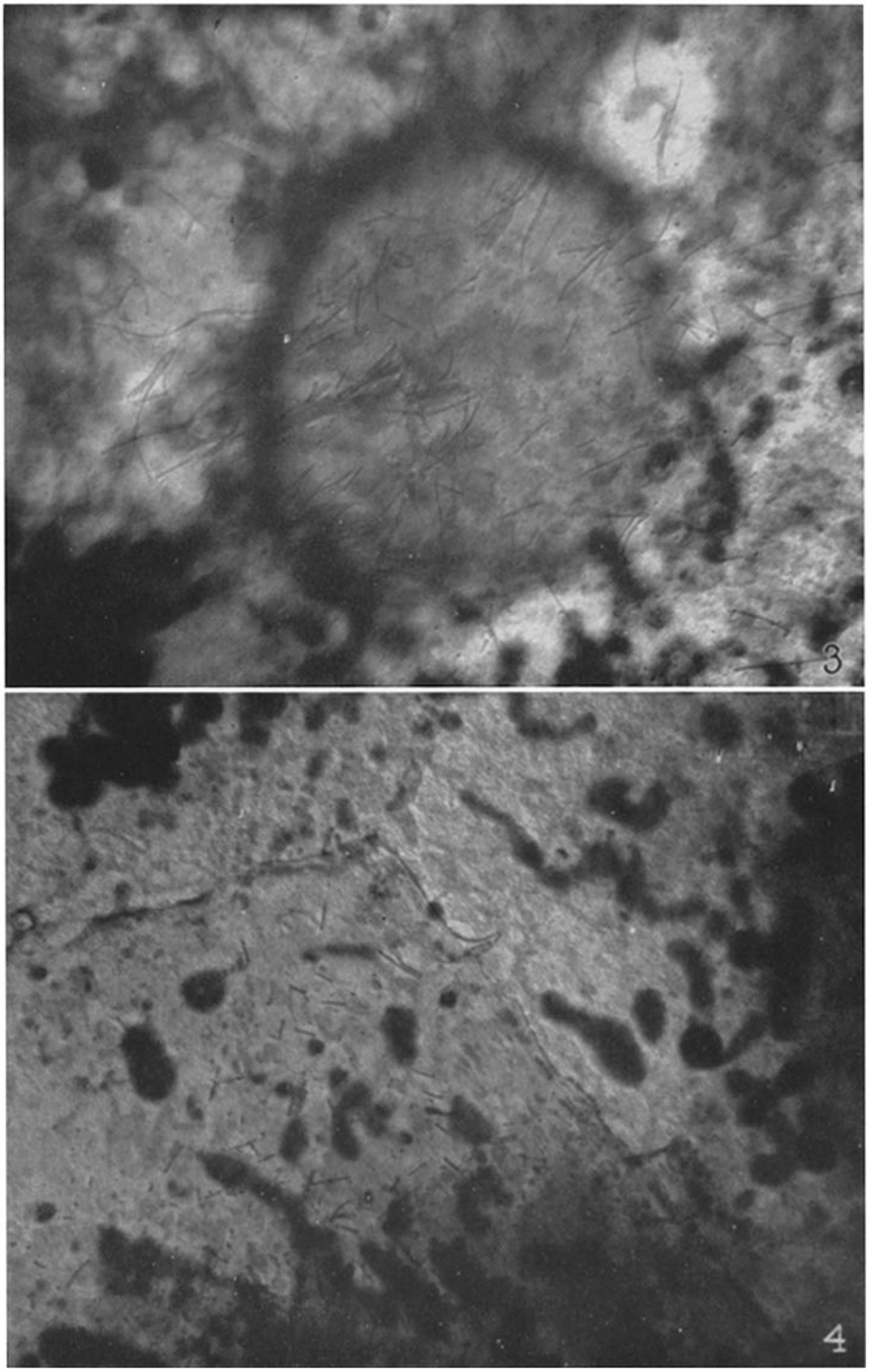


(Murphy and Bang: Electron microscope study of influenza-infected cell)

PLATE 14

FIG. 3. A Maximow tissue culture cell from the chorio-allantoic membrane of an embryo infected with influenza A (PR8). Typical filaments and spheres are seen in relief against both the nucleus and cytoplasm. This cell, otherwise, appears normal. × 6200.

FIG. 4. A similar preparation of chorio-allantoic membrane infected with swine influenza (V15). The intersection of three cells is shown. Only the lower left-hand one shows short filaments and spherical particles. No other abnormality is seen in this cell. ×6200.

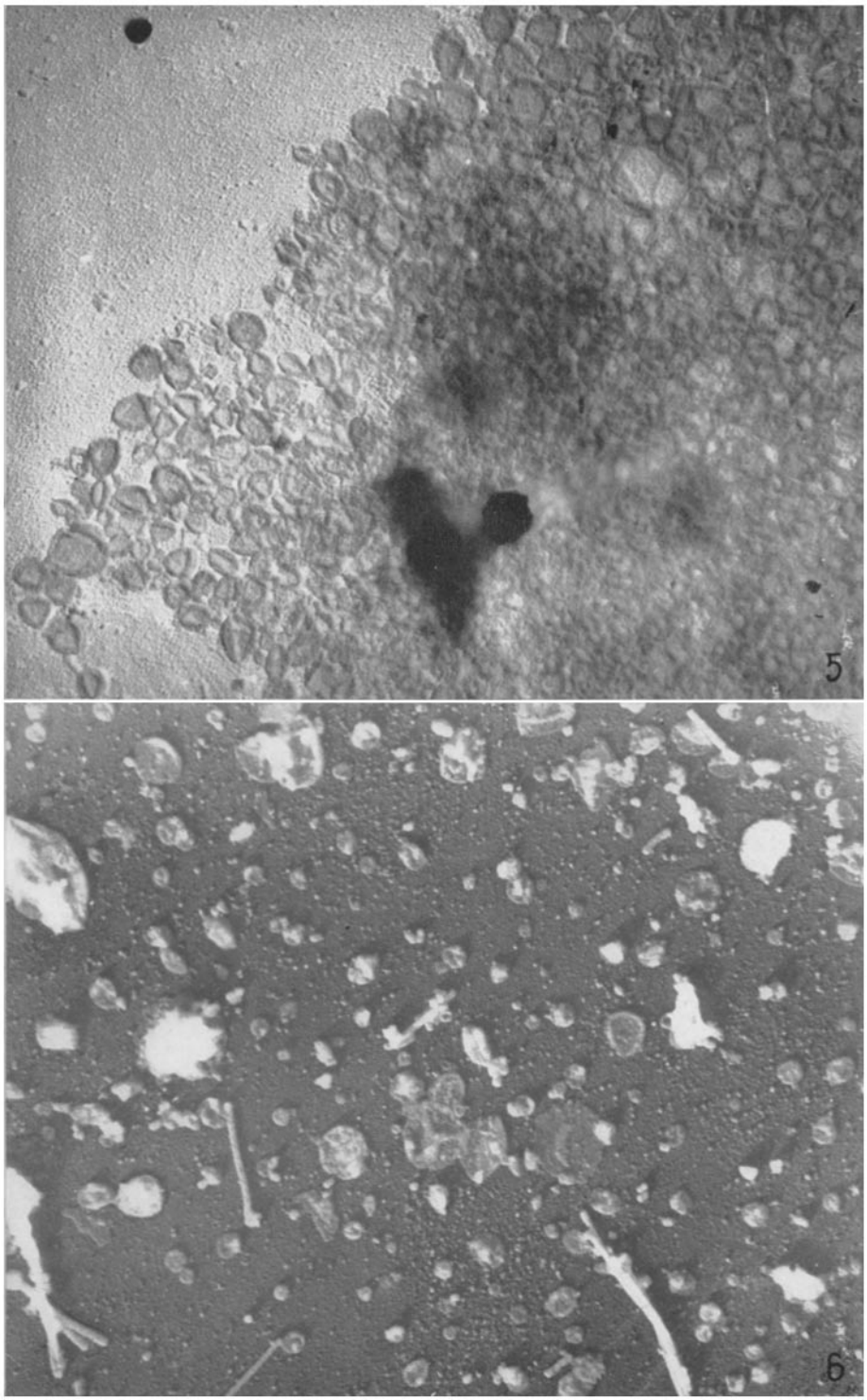


(Murphy and Bang: Electron microscope study of influenza-infected cell)

PLATE 15

FIG. 5. Area from tissue culture infected prior to *in vitro* cultivation of the cells. This shows a cell which seems to have completely degenerated into sacs. ×8900.

FIG. 6. Crude concentrated allantoic fluid from embryos infected for 22 hours with influenza A (PR8). This field was chosen because of the unusually large number of sacs. × 8900.

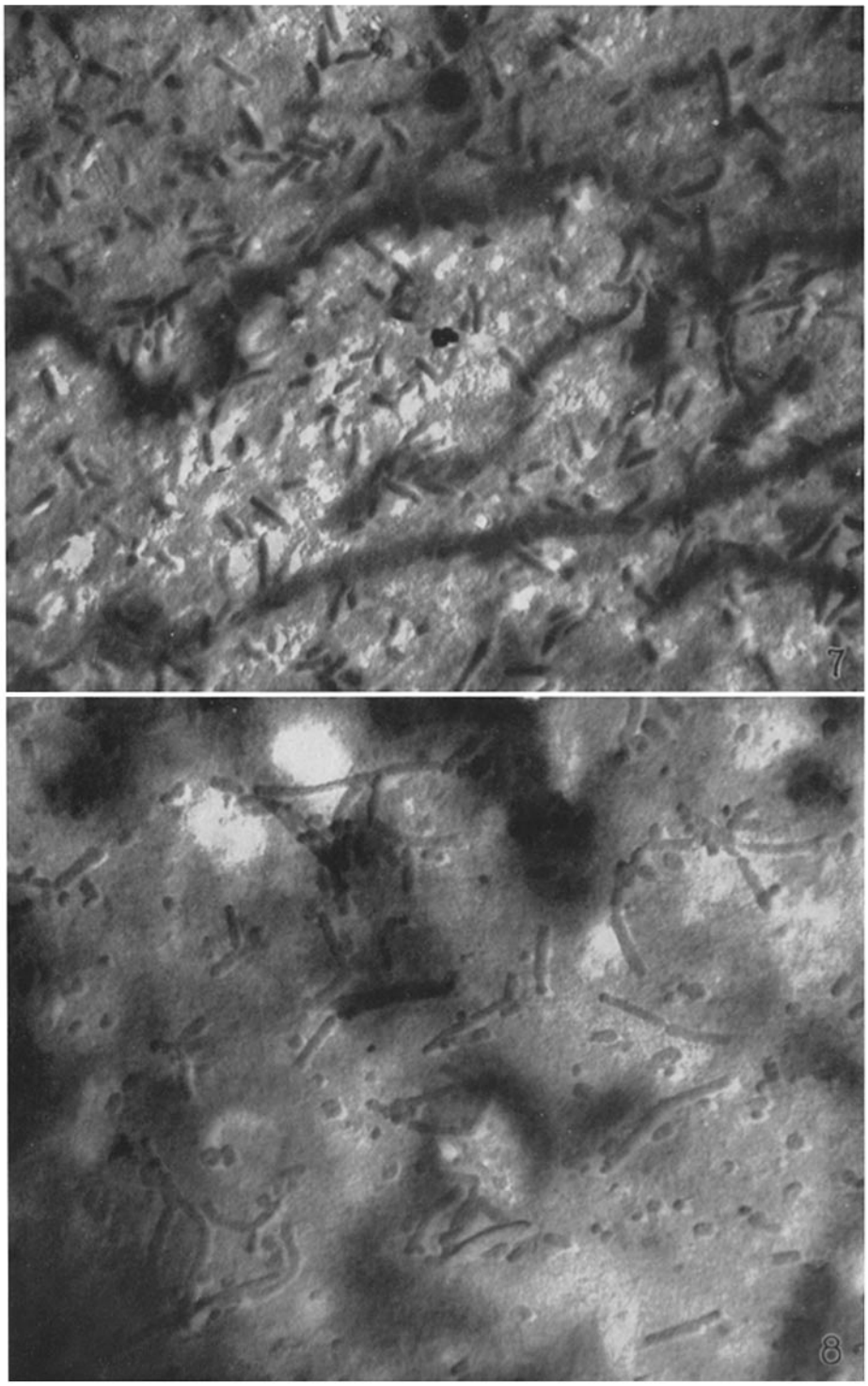


(Murphy and Bang: Electron microscope study of influenza-infected cell)

PLATE 16

FIG. 7. A roller tube preparation showing a normal 10 day chick chorio-allantoic membrane cell. "Normal" filaments are seen everywhere. In spite of the fact that this preparation was handled in a somewhat different manner than that shown in Fig. 8, it is included because it demonstrates how much normal filaments resemble those of influenza. × 16,300.

FIG. 8. A roller tube culture of chick chorio-allantoic membrane after 2 days *in vitro* and 6 hours of exposure to swine influenza (V15). Filaments with parallel sides and terminal nodules are seen. This preparation also shows "normal" filaments particularly in the right upper corner. There are several spherical particles in the photograph which probably come from the inoculum; they are dense and sharply outlined. × 16,300.

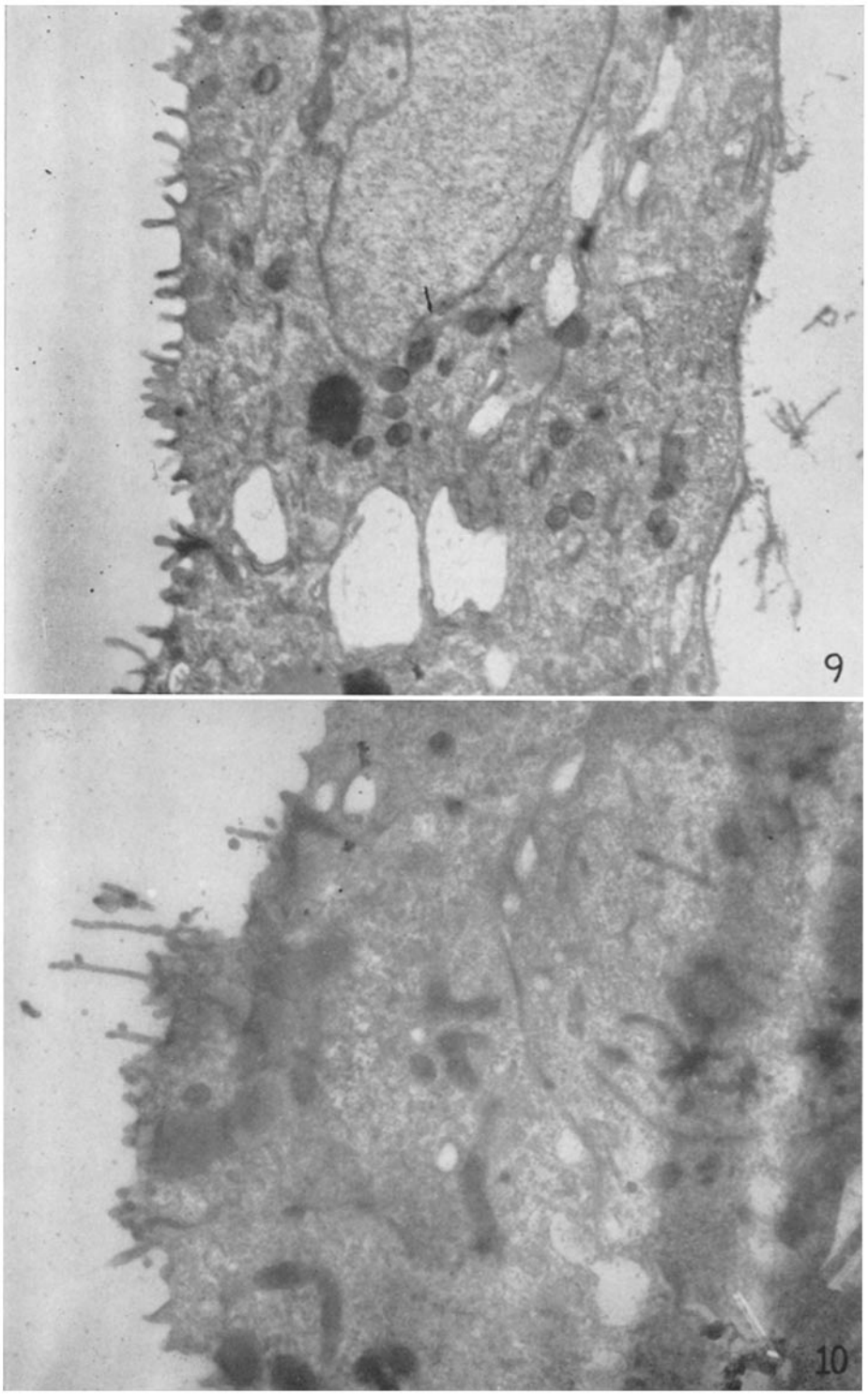


(Murphy and Bang: Electron microscope study of influenza-infected cell)

PLATE 17

FIG. 9. A section of 10 day old normal chick chorio-allantoic membrane showing filamentous projections along the surface. The variability and club-shaped character of these projections are well demonstrated. The occasional condensation of material at the tip of these projections can be distinguished from the terminal nodules by its diffuseness and irregular form. × 16,700.

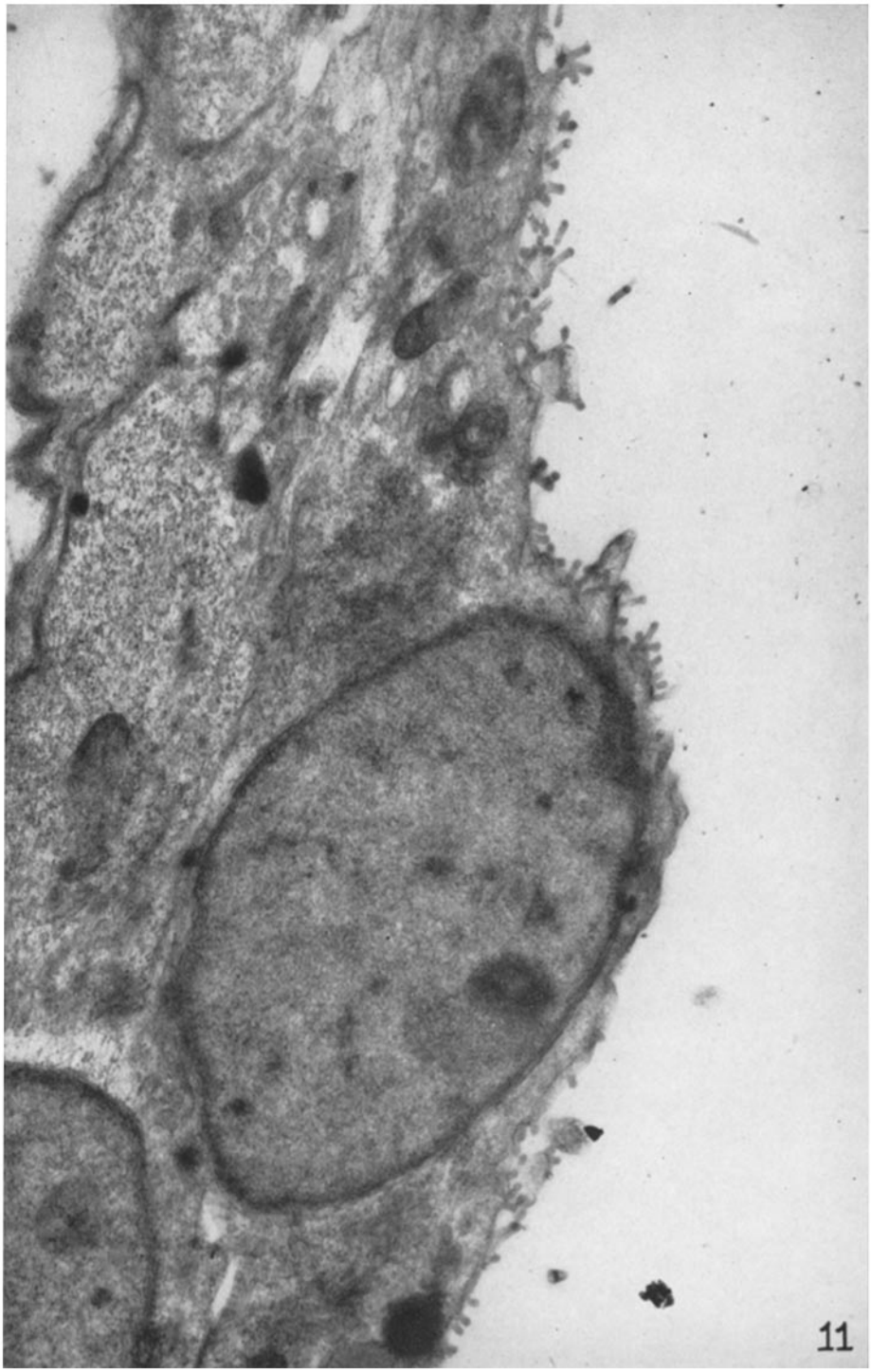
FIG. 10. A section through a 12 day old chick chorio-allantoic membrane infected 48 hours previously with influenza A (PR8). Filaments, spheres, and a segmented filament are seen. The nucleus on the right shows the change sometimes seen in infected cells. × 16,700.



(Murphy and Bang: Electron microscope study of influenza-infected cell)

PLATE 18

FIG. 11. A section through a 13 day old chick chorio-allantoic membrane inoculated 2 days previously with swine influenza (V15). Short filaments along the edge of the cell, with terminal nodules, are apparent. There are a few spherical particles present. The nucleus and cytoplasm appear normal. × 24,600.



(Murphy and Bang: Electron microscope study of influenza-infected cell)