THE RELEASE OF INFLUENZA VIRUS FROM THE INFECTED CELL

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(With Plates 5–10)

Studies of the growth cycle of influenza virus in the fertile egg in recent years (Hoyle, 1948, 1950; Henle & Henle, 1949; Burnet & Lind, 1951*a*, *b*) have shown that the infective virus particle is not reproduced in the host cell as such, but that on entry into the cell the particle appears to be broken down into smaller units which are reproduced in the cell. Fully infective virus does not reappear until 6 hr. later at a time coincident with the excretion of virus protein from the cell. The presence of virus protein in the cell can be recognized by its serological properties. Saline extracts of the chorioallantoic membrane made 4–5 hr. after infection contain red-cell agglutinin and give complement fixation with human convalescent sera, but the infectivity of such cell extracts is very low. The complement-fixing and red-cell agglutinating properties are largely present in separate particles and not in a single unit. By contrast, in the allantoic fluid 8 hr. after inoculation virus is present in a highly infective form and with red-cell agglutinin and complement-fixing antigen present as a single unit. When small pieces of chorioallantoic membrane suspended in allantoic fluid were examined in the dark-field microscope it was observed (Hoyle, 1950) that spherical and tubular protrusions were formed from the margins of the cells and that these became detached from the cell. It was suggested that the process of excretion of influenza virus from the cell was essentially a process of disintegration of the cell cytoplasm whereby portions of cytoplasm enclosed in cell membrane became detached from the cell. The infective particle was regarded as a fragment of the cytoplasm of the infected cell (Hoyle, 1950, 1953). Murphy, Karzon & Bang (1950), Murphy & Bang (1952), Eddy & Wyckoff (1950), Wyckoff (1951, 1953) studied the excretion of virus from the cell by means of the electron microscope and observed structures similar to those seen by Hoyle using the dark-field method. Wyckoff also concluded that the infective particle was a fragment of cell cytoplasm. The electron microscope gives high magnification and high resolution but has the disadvantage that material must be fixed and dried before examination. The dark-field method permits a study of living material and therefore gives a more reliable picture of the structures and of the sequence of events. The potentialities of the dark-field microscope are often underestimated by workers without experience of the method. The resolution attainable is considerably greater than with the ordinary light microscope, and particles which are below the limit of resolution may be clearly visible. In the author's earlier work the phenomena observed were represented by drawings, but with better equipment it has proved possible to photograph many of the stages of

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cytoplasmic fragmentation. The camera, however, is an inferior instrument to the human eye. Brownian movement may render it impossible to photograph an object clearly visible to the eye, and the resolution obtainable in a photograph is limited by the grain of the film. The latitude of the photographic film is also much less than that of the eye, so that it is impossible to show in the same photograph the structural details of an intensely illuminated object such as a cell and a much smaller less intensely illuminated virus filament.

TECHNIQUE OF DARK-FIELD MICROSCOPY

The microscope used in this work was equipped with a focusing dark-field condenser and three objectives, a low-power objective used for centring and locating the field, and two high-power oil immersion objectives, (1) a 3 mm. apochromatic objective working at a numerical aperture of 0·95 used for the photographic and much of the visual work, and (2) a 2 mm. parachromatic objective with variable iris diaphragm giving numerical apertures ranging from 0·25 to 1·25 and used for visual work at high magnification. The source of illumination was a 250 W. high-pressure mercury arc focused by means of a 3 in. diameter condenser to deliver a cone of light of maximum intensity to the dark-field condenser.

The photographic work presented great technical difficulties. Brownian movement in the specimens necessitated the use of very short exposures and called for the use of low initial magnification and high-speed film. Maximal resolution called for a high initial magnification and low-speed fine grain film. A compromise was therefore necessary. Most of the photographs presented in this paper were made with an objective of N.A. 0·95. A Beck photomicrographic camera was used giving a negative 1 in. in diameter on 35 mm. cinematograph film. A beam-splitting device enabled simultaneous visual examination and photographic recording to be made. An initial magnification of 220 diameters was used and the negatives were enlarged photographically to 1000 diameters. High-speed panchromatic film was used and developed with a fine-grain developer. Under these conditions exposures of $\frac{1}{5}$–$\frac{1}{10}$ sec. were satisfactory. When Brownian movement was not a difficulty fine-grain panchromatic film was used with a longer exposure. The photographic resolution attained was inferior to that obtained visually, but it seems doubtful if the results could be improved without a light source of much greater intensity.

CYTOPLASMIC FRAGMENTATION IN NORMAL CHORIOALLANTOIC MEMBRANE

The phenomenon of cytoplasmic fragmentation is not peculiar to the infected cell but may be seen in normal cells. If a small portion of the lining membrane of the allantoic sac of a 12-day developing egg is detached by scraping with a platinum loop, suspended in a drop of normal allantoic fluid, and examined in the dark-field microscope, protrusions are seen to develop from the margins of the piece of tissue. The great majority of these protrusions are spherical, but occasional tubular protrusions may be seen (Pl. 5, figs. 1–3). Tubular protrusions usually fragment into bead-like chains (Pl. 5, fig. 4). Both spherical and tubular protrusions break away from the cells and float off into the surrounding fluid. Many of the spherical

globules appear quite transparent in the dark field, but others appear opalescent suggesting the presence of particles on the borderline of dark-field visibility, while some show clearly visible particles in very vigorous Brownian movement. It is evident that the contents of the globules are fluid and not gelatinous in consistency, and they contain particles of varying size. Cytoplasmic protrusions are not formed merely from the torn marginal cells of the piece of membrane; they are formed from all the cells and become squeezed out to appear in large numbers along the edge of the tissue. After about 30 min. examination the area occupied by the detached globules becomes about equal to the area of the tissue, and it appears that the cells can detach about half their total volume of cytoplasm in this way. The formation of cytoplasmic protrusions is not accompanied at first by any other evidence of cell damage, and red cells which may be present in the preparation show no morphological changes. The detachment of globules from the cell does not rupture the cell membrane. Very often a second globule appears at the point from which the first originated, and if the first globule has not become detached the second may be forced into the interior of the first so that globules may be encountered which contain other globules inside them. These observations indicate that the protrusions have a surrounding membrane and that they contain fluid.

At a later stage, usually from 45 to 60 min. after commencement of examination the cells disintegrate, usually quite suddenly. Red cells present in the preparation become lysed, the tissue becomes transparent, the cell outlines disappear and the remaining cytoplasm streams out in the form of long filamentous processes. Numerous highly refractile fat globules escape from the cells, and on making contact with the surrounding fluid spread out into characteristic myelin figures (Pl. 5, fig. 5). These changes indicate destruction of the cell membrane with resulting death of the cells. It seems probable, however, that the earlier stage of formation of cytoplasmic protrusions is not so much the first evidence of cell damage as a normal physiological process, which occurs in the intact egg. This view is supported by the fact that spherical globules indistinguishable from those seen to appear from the cell margins in detached portions of membrane can be found in normal allantoic fluid.

CYTOPLASMIC FRAGMENTATION IN THE INFECTED CELL

If a detached fragment of the lining membrane of the allantoic sac of an egg infected with influenza virus is examined in the dark-field microscope, cytoplasmic protrusions are seen similar to those found with normal membranes. While no appearance has been seen in the infected egg which has not on occasion been seen with normal membranes, nevertheless there are certain quantitative differences. Thus the production of cytoplasmic protrusion in the intact infected egg appears to be more pronounced than in the intact normal egg. In the allantoic fluid of infected eggs when virus is being actively excreted from the cells spherical globules are much more numerous than in normal allantoic fluid and tubular and filamentous particles are seen which are rare in normal eggs. Minute spherical particles are present in great numbers. Infected allantoic fluid also contains moderately

numerous desquamated cells which may well be a result of very vigorous production of cell protrusions in the infected membrane.

In detached pieces of membrane from infected eggs tubular protrusions are very numerous, whereas in the normal egg the spherical type of protrusion is most frequent; and in the infected membrane tubular protrusions show less tendency to fragment into spherical units than in the normal egg. Appearances typical of infected membranes are shown in Pl. 6, figs. 6–11.

These differences are seen best in membranes examined when virus is being excreted from the cells. Infected membranes examined before excretion commences resemble those from normal eggs, with spherical protrusions dominating the picture and only a few tubular protrusions being seen. One of the main advantages of the dark-field method over the electron microscope is that it is possible to follow the sequence of events in the development of cell protrusions. Spherical cell protrusions develop rapidly and usually break away from the cells quickly so that spherical globules of varying sizes are discharged into the surrounding fluid. When, however, a large number of such protrusions are formed simultaneously they become closely packed, and those in the centre of the mass may become squeezed into a tubular form with a spherical swelling at the end. A protrusion which has adopted a tubular form in this way does not revert to the spherical form even if the compression by surrounding globules is removed; on the contrary, the tubule becomes progressively longer and narrower until ultimately it becomes so narrow that it can no longer be resolved and appears as a filament with a spherical globule at the end, an appearance suggesting a balloon on a string (Pl. 5, fig. 3). Often the terminal spherical globule breaks away leaving a tubule or filament with a rounded end. Tubular protrusions do not always progress to the filament stage; they often fragment into chains of spherical or ovoid units. There appears to be a critical tubule diameter of about 0·5 μ at which this change is most prone to occur. The membrane surrounding the tubule seems to be under some degree of tension and to be elastic. A sudden movement in the surrounding fluid sets up a wave which passes along the tubule producing alternate points at which the tubule is wider and narrower than the average diameter. If the amplitude of the wave is such that the walls of the tubule make contact at the points of narrowing then the tubule fragments into a bead-like chain. This is most likely to occur when the diameter of the tubule is about 0·5 μ. If the diameter of a tubular protrusion becomes less than 0·5 μ without fragmentation occurring, then the tendency is for a filament to be produced. The individual units into which a tubule may fragment usually separate quickly. Since some twenty to fifty small spherical units of diameter about 0·5 μ may result from the fragmentation of a single tubular protrusion the numbers of spherical units of this size discharged from an infected membrane into the allantoic fluid far exceeds the numbers of spherical globules of larger size. Since the fragmentation of tubular protrusions usually results from a sudden movement of the surrounding fluid (it can sometimes be induced by tapping the stage of the dark-field microscope) it is very difficult to obtain a photograph of this stage, but some successes are shown in Pl. 6, figs. 9–11.

Two strains of influenza virus A have been used in this work. The D.S.P. strain of virus A first isolated in 1943 is a typical old laboratory strain of which the

infective units have a predominantly spherical morphology. The Burch strain is an A′ strain first isolated in 1949 and showing in addition to spherical particles numerous filaments of the types described by Mosley & Wyckoff (1946), Heinmetz (1948) and Chu, Dawson & Elford (1949). No essential differences were observed in the development of cytoplasmic protrusions from chorioallantoic membrane infected with the two strains. In each case the sequence of events was the same, but with the D.S.P. strain there was a greater tendency for tubular protrusions to fragment into chains of small spherical particles, while with the Burch strain tubular protrusions more frequently contracted into filaments without undergoing fragmentation.

The phenomena just described undoubtedly occur in the intact infected egg. In allantoic fluid from an infected egg desquamated cells may be found which show all types of cytoplasmic protrusion and the sequence of events described above can often be followed with such cells. Pl. 7, fig. 12, shows a group of desquamated cells found in infected allantoic fluid, and the development of spherical protrusions, while Pl. 7, figs. 13 and 14, show cells with tubular and filamentous protrusions.

CYTOPLASMIC PARTICLES IN INFECTED ALLANTOIC FLUID

All types of cytoplasmic protrusion may become detached from the cells at any stage, with the result that allantoic fluid from infected eggs shows on dark-field examination a wide variety of particles, some being of very complex morphology. Spherical particles of all sizes range from minute particles on the borderline of dark-field visibility up to large globules of 2–5 μ diameter. Tubular and filamentous particles are also seen and some may show a tubular form with spherical swellings. Although these particles are in very active Brownian movement satisfactory photographs can often be obtained by focusing either on the surface of the slide or the undersurface of the cover-slip and waiting for suitable particles to adhere. At the moment when a particle adheres to the slide Brownian movement ceases and a photograph can be obtained. Once particles have become firmly adsorbed to the slide their light scattering is reduced and photographs are less satisfactory. Pl. 7, figs. 15–17, show tubular and filamentous particles photographed in this way.

If allantoic fluid from an infected egg is allowed to stand in the refrigerator for 24–48 hr. certain changes occur in the type of particles present. The number of large spherical globules becomes greatly reduced and spherical particles appear which are more refractile, smaller in size and appear solid. It seems probable that these are developed by a process of contraction of the large globules due to loss of water. The disappearance of the larger globules is, however, partially explained by the fact that many of them burst. This has been seen to occur under the microscope when a large globule comes into contact with the slide. Tubular and filamentous particles also undergo striking changes on standing. Tubular units are rarely seen in allantoic fluid which has been stored for 48 hr. Filamentous forms in fresh allantoic fluid are usually flexible and wavy (Pl. 7, fig. 16); on storage they become apparently narrower in diameter, shorter in length and appear as rigid rods which do not wave under the influence of Brownian movement. Such rigid rods are often bent at sharp angles and the angle does not alter under Brownian

impact. Pl. 8, figs. 18 and 19, show dark-field photographs of such filaments and, Pl. 8, fig. 20, an electron microscope picture of the same material. The development of rigidity on standing is probably a result of loss of water and consequent close aggregation of protein units.

The majority of the spherical particles detached from the infected cell have an initial diameter of the order of 0·5 μ since they are produced by the fragmentation of tubular protrusions of this diameter. On standing, these particles contract in the same way as is seen with larger globules, probably as a result of loss of water, with the result that most of the spherical particles found in infected allantoic fluid have a particle size of 0·1–0·15 μ, the size of the elementary bodies seen in electron microphotographs of virus suspensions. Hoyle, Reed & Astbury (1953), in electron microscope studies of influenza virus elementary bodies, found that such bodies frequently showed a somewhat hexagonal outline which was possibly due to hexagonal close packing of smaller units within them.

If the elementary body suspension was shaken with ether the bodies were disrupted with the release of numerous small particles of which the great majority had a diameter of about 12 mμ. This disruption appears to take place at the ether-water interface. The first stage appears to be an accumulation of the bodies in agglutinated masses at the interface followed by disintegration as lipid is removed by the ether. Pl. 9, fig. 21, is an electron microscope picture of a preparation of D.S.P. virus elementary bodies which shows the hexagonal form of some of the particles. Pl. 10, fig. 22, shows the same preparation fixed with osmic acid after a brief period of ether treatment. The elementary bodies have become agglutinated collapsed and flattened, and are seen to contain numerous small particles.

DISCUSSION

The processes of formation of cytoplasmic protrusions described here is not peculiar to the cells of the chorioallantoic membrane but may be seen with a wide variety of cells. Zollinger (1948), using phase-contrast microscopy, described the phenomenon in detail and gave references to previous observations. The phenomenon has been seen with a wide variety of normal cells and with malignant cells. It is not seen with squamous cells. Zollinger noted that the phenomenon only occurs with living cells and ceases when the cells die. It is possible that the production of cytoplasmic protrusions may be of great physiological importance; for example, it may explain the mechanism of glandular secretion.

All previous observations have related to the production of spherical protrusions only; the tubular type of protrusion does not appear to have been seen. Nevertheless, it is clear that there is no essential difference between the two types of protrusion. The tubular protrusion commences as a sphere but develops the tubular form as a result of compression by surrounding globules. Once the tubular form has developed it does not revert to the spherical except as a result of fragmentation into beads. Tubular protrusions tend to develop more readily in the infected egg than in the normal. This may be due to the simultaneous production of protrusions in great numbers from the cell margins at the time of excretion of virus and may well result from damage to the cell membrane by the virus mucinase.

There can be no doubt that the infective properties of allantoic fluid from virus-inoculated eggs are carried by the elementary bodies and filaments which can be found in the fluid. By means of the dark-field microscope it is possible to follow the complete sequence of events whereby these particles are produced from cytoplasmic protrusions. Filaments are produced from detached tubular protrusions by loss of water, and elementary bodies are produced by the fragmentation of tubular protrusions. Larger particles are also formed which are derived from the larger spherical types of protrusion but these are relatively scarce in comparison with the smaller particles. Most of the electron photomicrographs of influenza virus which have been published in recent years give a false impression of homogeneity of size due to the use of preliminary differential centrifugation. If crude allantoic fluid is adsorbed with laked red cells and an electron photomicrograph taken, the cells are seen to adsorb, in addition to elementary bodies and filaments, many particles of much larger size. This is well seen in a photograph by Chu *et al.* (1949).

The dark-field microscope and the electron microscope are both required for a complete study of the process of release of influenza virus from the infected cell. The dark-field microscope is most satisfactory for the study of the earlier stages of cytoplasmic fragmentation and for the study of the sequence of events in living material, while the electron microscope is required for the later stages when the particles have become smaller and have fallen below the limit of resolution with visible light. The internal structure of the particles can be studied only with the electron microscope.

The electron photomicrographs of Wyckoff (1953) give an excellent picture of the later stages of development of virus particles from filamentous cytoplasmic protrusions, but the earlier stages of cytoplasmic protrusion formation are not seen, probably because the larger types of protrusion are too delicate to withstand the process of fixation and drying. Wyckoff did not encounter protrusions from normal membranes and considered the phenomenon to be a result of cell damage. However, Murphy & Bang (1952) found filamentous cell protrusions in both normal and infected membranes. Their pictures also show some large sac-like bodies which appear to correspond to the large spherical protrusions seen by the dark field method.

SUMMARY

1. When detached portions of the chorioallantoic membranes of normal fertile eggs are examined in the dark-field microscope spherical and tubular cytoplasmic protrusions are seen to develop from the margins of the cells and to become detached from them. This is probably a normal physiological phenomenon which occurs to some extent in the intact egg.

2. Similar cell protrusions occur from chorioallantoic membranes of eggs infected with influenza virus. The phenomenon appears to be more pronounced in the infected egg than in the normal egg and in particular tubular cytoplasmic protrusions are much more frequent in the infected egg than in the normal.

3. Tubular cytoplasmic protrusions may fragment into small spherical particles

or may contract into filaments. All types of cell protrusion become detached from the cells and undergo contraction in size probably as a result of loss of water. The infective elementary bodies and filaments present in the allantoic fluid of eggs infected with influenza virus are derived from the cells in this way.

4. It is concluded that the infective particle of the influenza virus is a fragment of the cell cytoplasm and consists of a closely aggregated mass of virus protein enclosed in cell membrane.

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EXPLANATION OF PLATES

PLATE 5

Cytoplasmic protrusions from normal chorioallantoic membrane. × 1000.

Fig. 1. Production of spherical protrusions and detachment from the cells.
Fig. 2. Development of a tubular protrusion.
Fig. 3. Thin tubular protrusion with terminal globular swelling.
Fig. 4. Tubular protrusion fragmenting into bead-like chain (left centre).
Fig. 5. Stage of total tissue disintegration with formation of myelin figures.

PLATE 6

Cytoplasmic protrusions from infected chorioallantoic membrane. × 1000.

Fig. 6. Early stage in the development of a tubular protrusion as a result of compression by surrounding spherical globules. Note agglutinated red cells on right. Burch virus.
Figs. 7, 8. Spherical, tubular and filamentous protrusions. Burch virus.
Fig. 9. Tubular protrusion fragmenting into bead-like chain. Centre. D.S.P. virus.
Figs. 10, 11. Tubular protrusions, some showing fragmentation. Burch virus.

PLATE 7

Detached cells and particles in infected allantoic fluid. × 1000.

Fig. 12. Group of detached chorioallantoic membrane cells showing development of spherical protrusions. Burch virus.
Fig. 13. Cell showing tubular protrusion. Burch virus.
Fig. 14. Cell with filamentous protrusions. Burch virus.
Fig. 15. Filamentous protrusion of complex morphology immediately after detachment from the cell seen lower right. Burch virus.
Fig. 16. Chorioallantoic membrane cell and detached flexible tubular protrusion. D.S.P. virus.
Fig. 17. Flexible filament with two spherical swellings. Burch virus.

PLATE 8

Virus particles in allantoic fluid stored for 24 hr.

Figs. 18, 19. Elementary bodies and filaments. Note the rigid appearance of the filaments and sharp angular bends. Burch virus. × 1000. Dark field.
Fig. 20. Electron microscope photograph of the same material as fig. 19. Note filaments bent at sharp angles and one terminating in a series of spherical swellings. Burch virus. × 27,000. Cr. shadowed.

PLATE 9

Fig. 21. Electron photomicrograph of sample of D.S.P. virus partially purified by adsorption and elution from red cells. Note that some of the elementary bodies, indicated by arrows, show a roughly hexagonal outline. Many smaller particles are also present. × 25,000. Cr. shadowed.

PLATE 10

Fig. 22. Same preparation as fig. 21 after a brief period of ether treatment. Elementary bodies are agglutinated, collapsed and flattened, and show commencing disintegration. Some of the particles retain a roughly hexagonal outline and show numerous small particles within them. × 30,000. Cr. shadowed.

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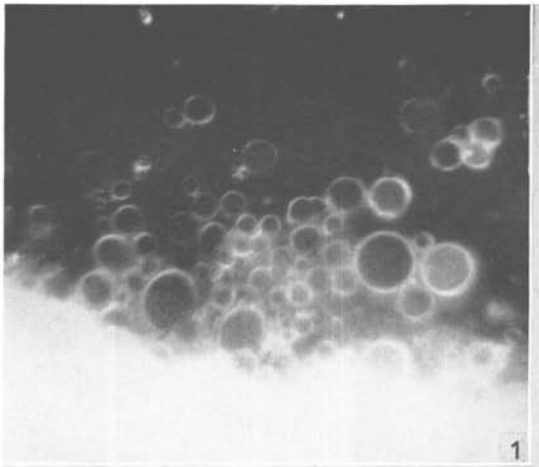

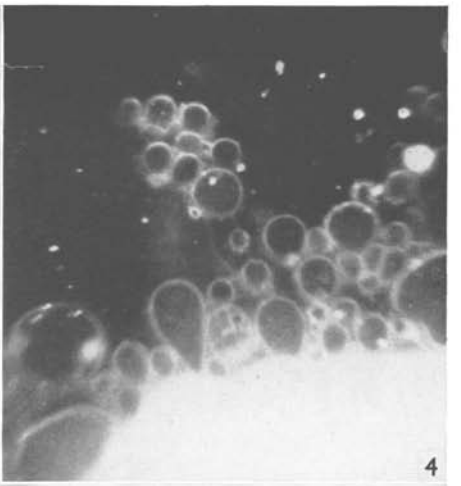

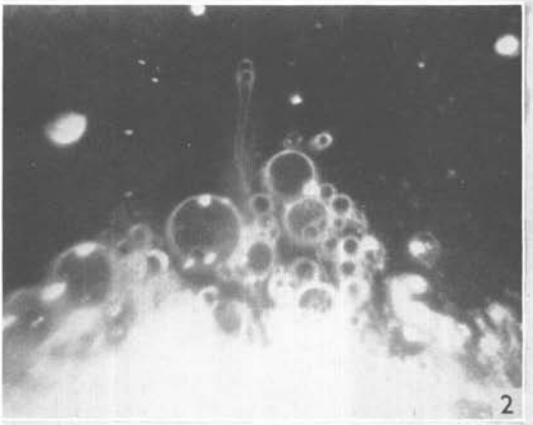

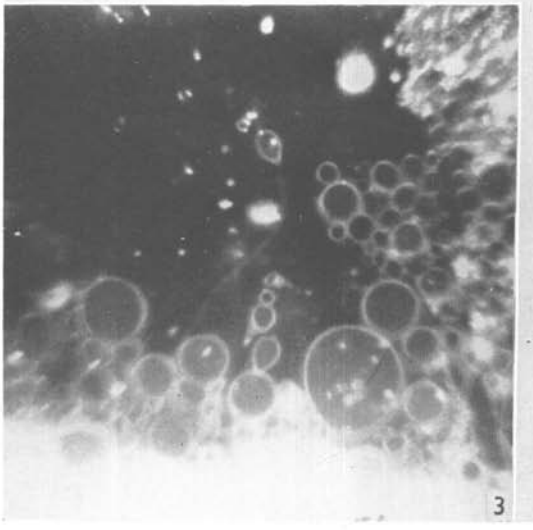

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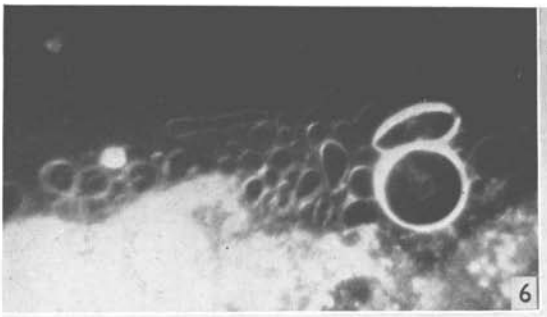

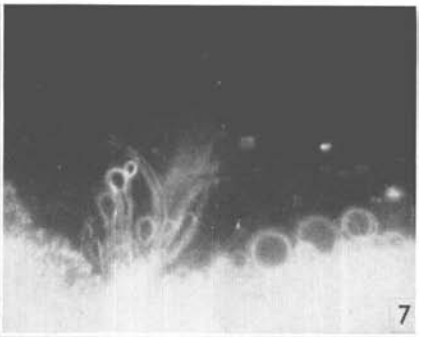

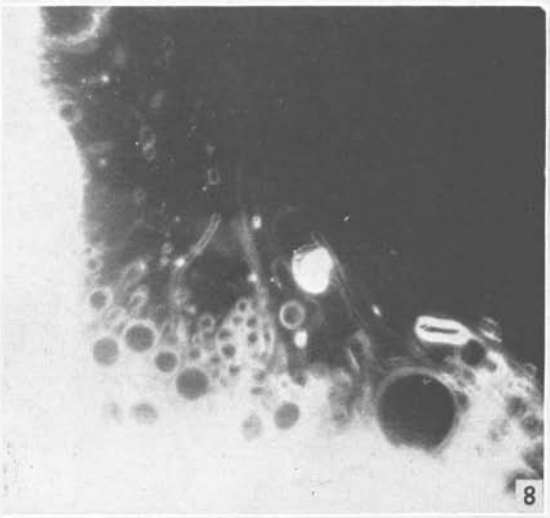

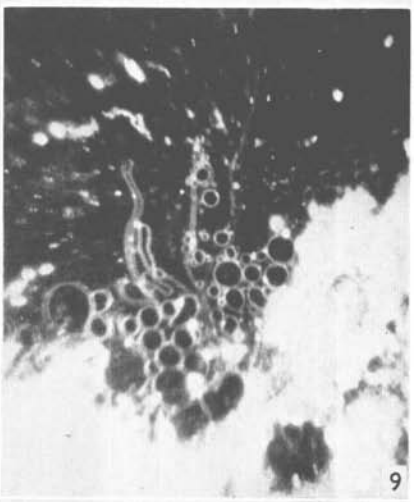

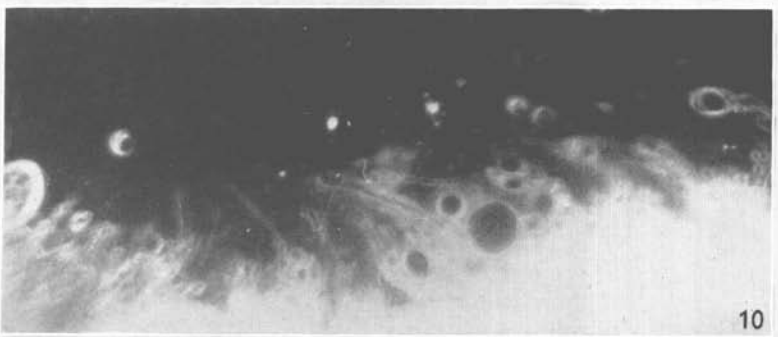

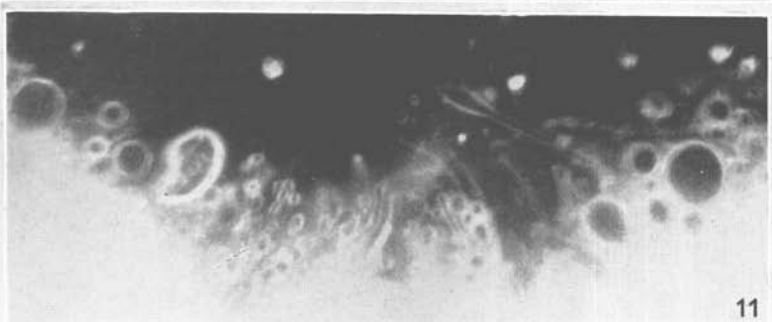

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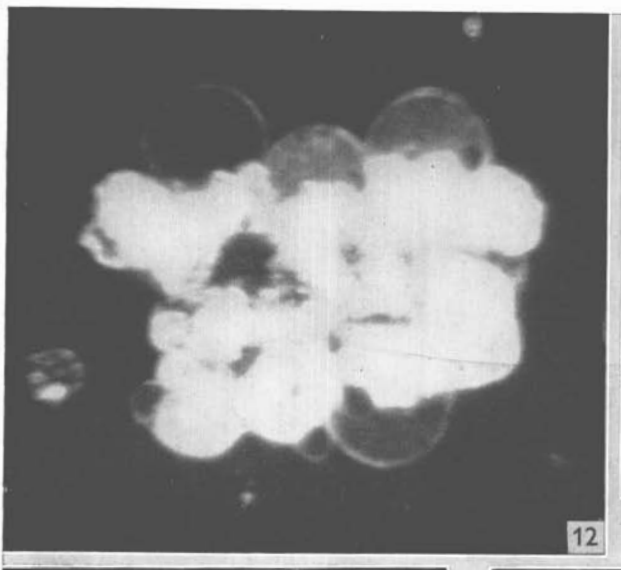

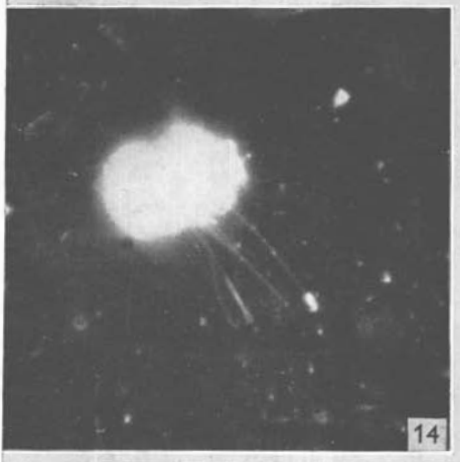

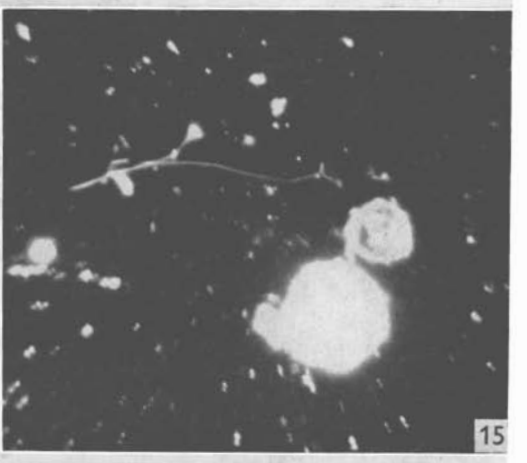

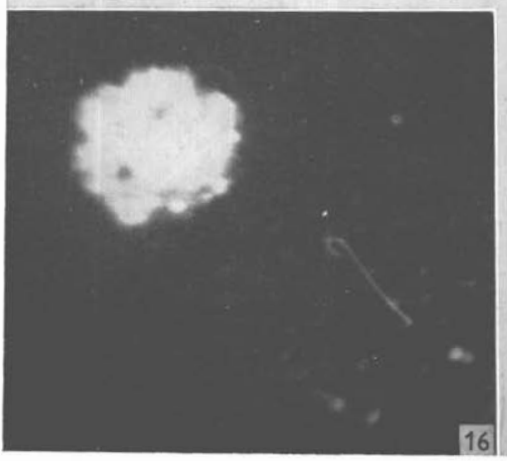

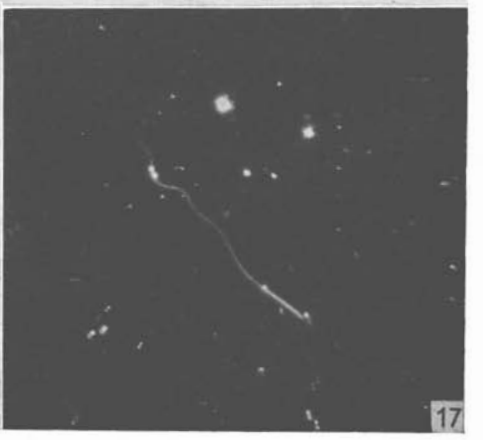

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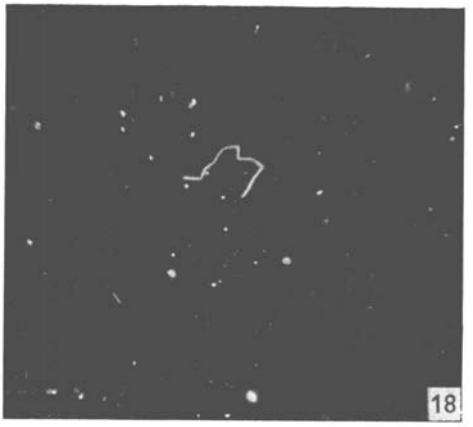

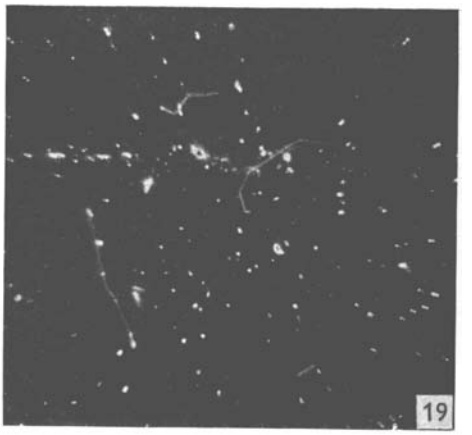

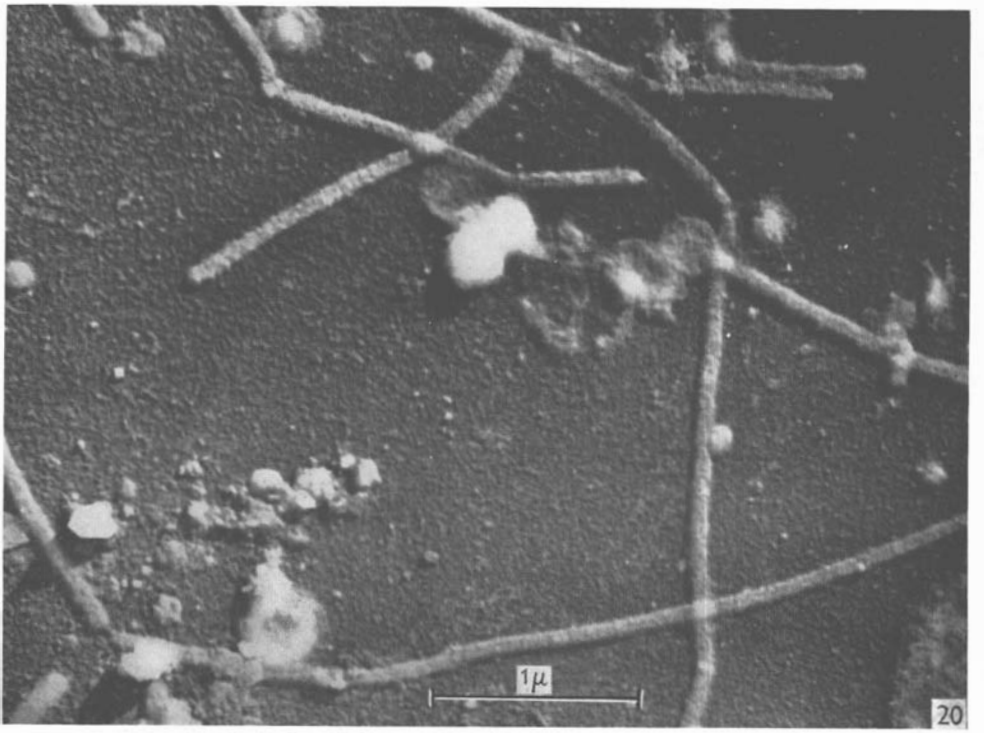

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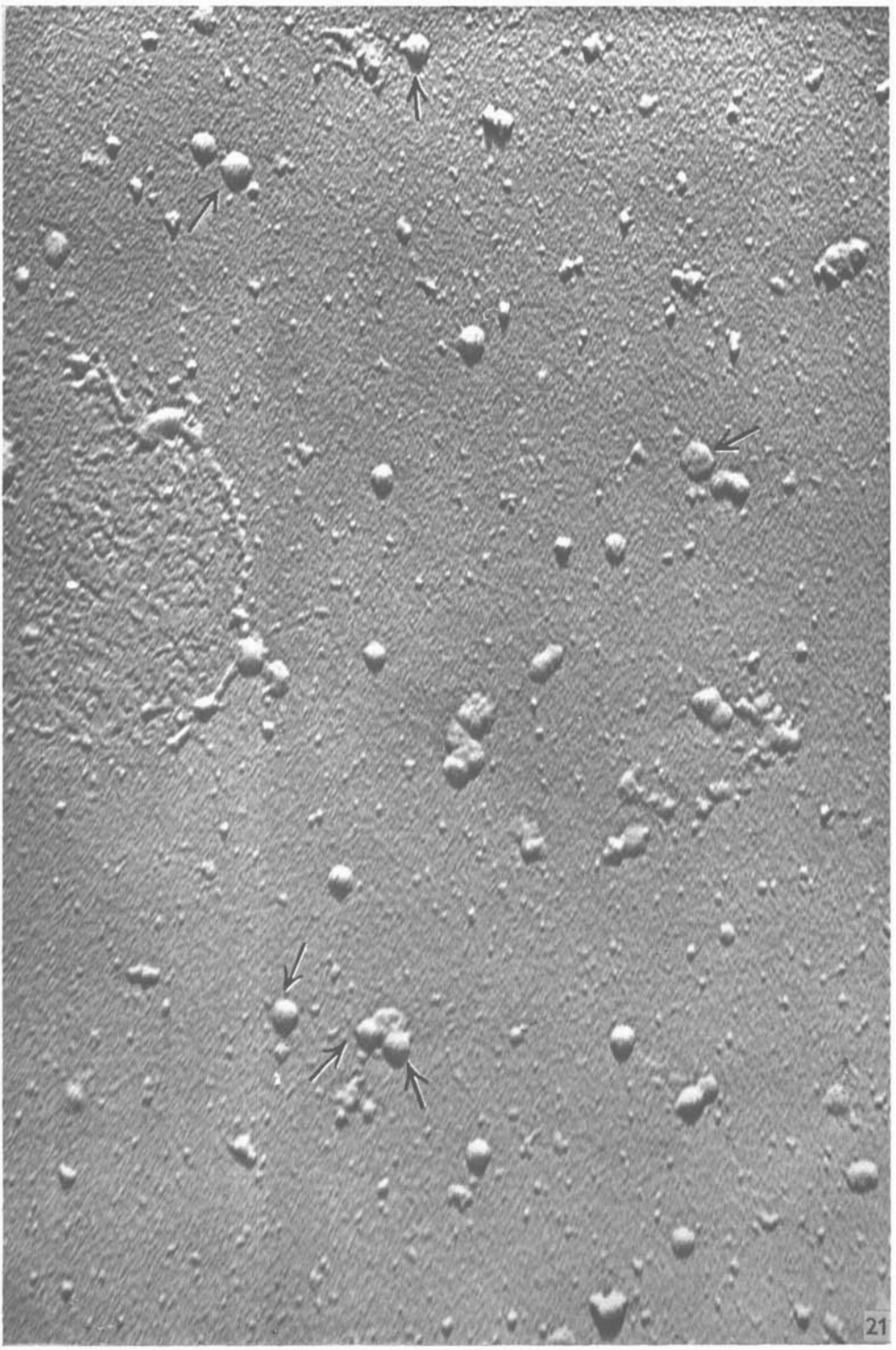

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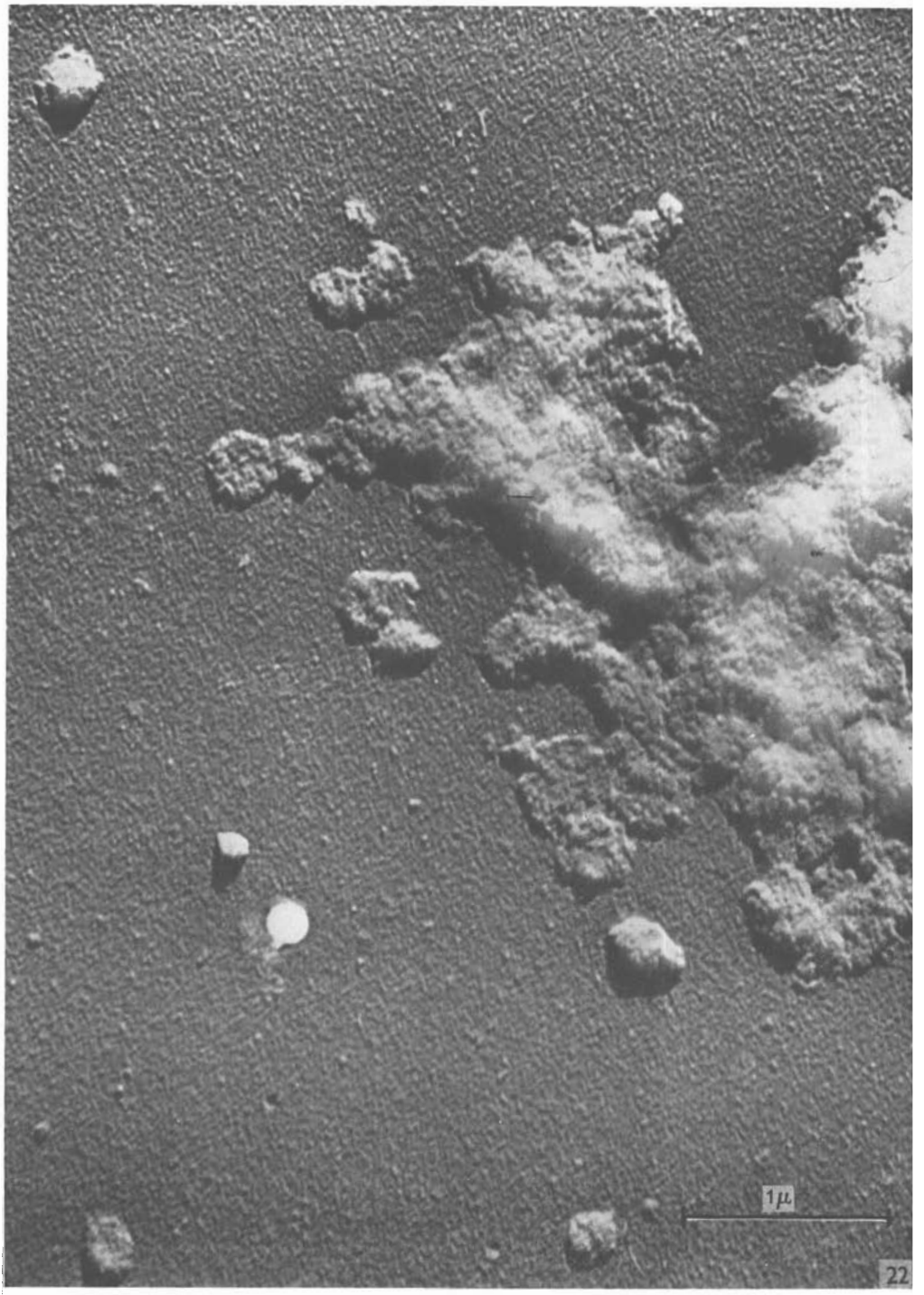

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