ELECTRON MICROSCOPY OF EARLY CYTOPLASMIC CHANGES DUE TO INFLUENZA VIRUS*

By CARL G. HARFORD, M.D., ALICE HAMLIN, AND ESTHER PARKER

(From the Division of Infectious Diseases of the Department of Medicine, David P. Wohl, Jr., Memorial Hospital, Washington University School of Medicine, St. Louis)

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We have already reported that cytoplasmic inclusion bodies are present in the early bronchial lesion due to influenza virus (1).[1] This finding was of interest because inclusion bodies had not been widely recognized as a characteristic of the lesion due to this virus and also because of the possibility that these structures might consist of colony-like aggregations of viral particles. The present investigation has been carried out by use of recently developed methods for examination of tissue sections in the electron microscope. These techniques appeared to offer an excellent way to confirm the existence of the inclusions and to resolve structural details sufficiently to visualize viral particles within the inclusions. Relationships between the inclusions and other cellular components have also been studied in order to gain further information concerning the environment in which the virus grows.

Because the electron microscope reveals many details of structure that are invisible by light microscopy, it is often difficult to interpret what is seen in electron micrographs. The present study represents an effort to overcome this difficulty by confining observations to one group of cells, the bronchial epithelium, and by comparing infected with non-infected cells under conditions in which alterations due to inoculation were minimal.

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[1] Since the publication of our paper describing cytoplasmic inclusion bodies due to influenza virus (1), Dr. F. O. MacCallum of London, England, has called our attention to work (2) in which bronchial smears of mice infected with influenza virus showed cytoplasmic bodies which probably correspond to the ones we have observed. Also, Dr. Thomas G. Ward has kindly allowed us to examine a thesis (3) describing and illustrating cytoplasmic inclusions in sections of bronchial epithelium of mice infected with influenza virus and in which it is evident that the inclusions were essentially identical with those we have described. Dr. Harold S. Ginsberg has directed us to a report (4) in which cells of the bronchial epithelium of chick embryos infected with influenza virus were found to contain cytoplasmic granules thought possibly to be microcolonies of virus.

The results have confirmed the observation of cytoplasmic inclusion bodies in this infection and have shown that the bodies contain many particles of a size approximately that expected of viral particles. No constant relationship has been found between the inclusions and other structures of the cytoplasm, but changes in the endoplasmic reticulum have been noted.[2]

Methods and Materials[3]

Strains of Virus.—Weiss and swine strains of mouse-adapted Type A influenza virus were the same as those used in previous studies (1). A PR8 strain of mouse-adapted Type A virus was obtained from the American Type Culture Collection. Two unadapted Type A prime strains (47-2 and 47-3) were kindly furnished by Dr. Goronwy Broun of St. Louis University. These strains had been isolated in eggs from patients of Dr. Broun and had been maintained only by passage in eggs.

Preparation and Administration of Viruses.—Mice[4] were infected by inhalation of aerosols containing the viruses. Techniques were the same as those previously employed for aerosols of pneumococci (7, 8) except that a glass nebulizer (DeVilbiss 44) was used. The chamber was a 5 gallon glass jar.

For serial passage of each mouse-adapted strain, the mice were allowed to inhale an aerosol of a 10 per cent suspension of infected mouse lung for 1 hour. After 72 hours, the lungs of the exposed mice were removed aseptically, ground in a mortar with sand, and suspended in 10 times their weight of 0.85 per cent sodium chloride containing 10 per cent inactivated horse serum. In most experiments, each milliliter of this diluent contained also 100 units of penicillin and 0.2 to 0.25 mg. of streptomycin. After centrifugation and culture for sterility, the supernatant fluid was stored in a dry-ice box. In each experiment, the fluid was administered to mice by aerosol for a period of 3 hours.

Passage of the unadapted strains was carried out in our laboratory by intra-allantoic injection of embryonated eggs. The viral content of the allantoic fluid was measured by hemagglutination on the day of harvest and the fluid was cultured for sterility before being stored in the dry-ice box. In the experiments, the undiluted fluid was administered to mice for 3 hours by the same method as for the adapted strains. Sections were prepared at the times noted in Table I. No macroscopic lesions were observed.

Controls.—In a previous investigation, it was found that the intrabronchial injection of fluids such as physiological saline, broth, or serum caused a lightly staining, swollen appearance of cells of the bronchial epithelium and also induced a small amount of acute inflammation in adjacent tissues (8). However, these changes due to the injection of diluent did not occur after inhalation of aerosols, and hence this method of inhalation by unanesthetized mice was adopted for infection of lungs to be examined by electron microscopy. Also, mice were allowed to inhale the virus for as long as 3 hours in order to infect as many cells of the bronchial epithelium as possible.

For control of the method of inoculation, sections were prepared from mice previously exposed for 3 hours to aerosols containing heat-killed virus. 10 per cent suspensions of mouse lung infected with the Weiss or PR8 strains of virus were made as for experimental infection

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[2] Preliminary reports of these findings have been made (5, 6).

[3] We should like to express our appreciation for the indispensable aid given by Dr. Edward W. Dempsey in carrying out these methods. Thanks are due also to Dr. Betty Geren for help given during the early stages of the work.

[4] Mice were obtained from Rockland Farms, New City, New York, and from Eldridge Rabbitry, St. Louis, Missouri.

except that they were heated to 56°C. for 1 hour. Another non-infectious 10 per cent suspension of pulmonary tissue was made from normal lung in the same manner except that it was not subjected to heat. The preparations were centrifuged, cultured for sterility, and stored in the dry-ice box until used. Sections were prepared as from normal and infected lungs.

Electron Microscopy.—The mice were killed with ether and immediately after death the left lobe of the lung was excised. The lobe was placed on a cork under a dissecting microscope and transverse slices about 1 mm. thick were made with a razor blade slightly below the point of entry of the bronchus into the lung. Examination of the cut surfaces revealed cross-sections of the larger intrapulmonary bronchi and further cuts were carried out so that most of the tissue surrounding a bronchus was trimmed away. The resulting blocks of fresh tissue were 1 to 2 mm. in each dimension and consisted mainly of small segments of intrapulmonary bronchi. The blocks were immersed in fixative immediately after cutting.

The fixative of buffered osmium tetroxide (9) was prepared freshly on the same day as it was used, and the pH was adjusted to 7.4–7.5. In the initial experiments, the blocks of tissue were allowed to remain in the fixative for 4 hours at room temperature. Subsequently, fixation was carried out for longer periods, usually 24 hours in the refrigerator, and it was found that a clearer visualization of intracellular membranes resulted (10). In two experiments, fixation was limited to 20 minutes. Blocks of tissue were imbedded in methacrylate (11) and a ratio of 1 part of methyl methacrylate to 2.5 to 3 parts of butyl methacrylate was used.

In order to locate cells of the bronchial epithelium easily in the electron microscope, the blocks of imbedded tissue were trimmed with a razor blade under a dissecting microscope until nearly all pulmonary tissue was removed except a bronchus or a portion of one. In many instances also, preliminary orientation was aided by light microscopy (12).

Sections were prepared by cutting with glass knives (13)[5] in a microtome designed to cut sections at a thinness of approximately 0.05 μ (14).[6] The microtome was equipped with an improved knife holder (15).[7]

As they were cut, sections were floated on the surface of a solution of 25 per cent acetone or 2.5 per cent *n*-octyl alcohol in water and these solutions were held to the edge of the knife by a small paraffin boat. Thinness of sections was estimated by observation of interference colors (16) and sections with a light yellow color were considered most suitable. Silver colored sections were often found to be too thin to be satisfactory because continuity of some intracellular membranes was lost.

For support of the sections, 150 mesh copper grids[8] were coated with a solution of 0.2 per cent parlodion in secondary butyl acetate.

The sections were examined in an RCA electron microscope, type EMU2C. Photographs were taken at a magnification of 2000 to 6000 times and further magnification was effected when prints were made.[9] In order to insure reproducibility of observations, more than 700 photographs were prepared.

RESULTS

Uninfected Bronchial Epithelium

To evaluate the significance of cellular changes in the bronchial epithelium of mice infected with virus, it was necessary to carry out an initial study of

[5] Obtained from Acme Glass Co., Boston.

[6] Minot rotary microtome, International Equipment Co., Boston.

[7] This device was made by Mr. William Fuchs.

[8] Athene grids, Smethurst High-Light Ltd., Sidcot Heaton, Bolton, Lancashire, England.

[9] Prepared by Mr. Cramer Lewis of the Division of Illustration.

normal epithelium. Furthermore, although inhalation of non-infectious diluents did not induce any cellular lesions that could be detected by light microscopy, there was a possibility that such changes might be visible by electron microscopy. Therefore, mice were allowed to inhale aerosols of heat-inactivated virus for 3 hours and sections were prepared for electron microscopy at intervals of 12, 14, 16, and 23 hours. An extract of normal tissue, ground, suspended, and centrifugalized like that containing virus, as described under Methods and Materials, was administered similarly as an aerosol for 3 hours and sections were prepared 23 hours later. No significant difference was detected between the bronchial epithelium of normal mice and that of mice inhaling these aerosols, and the following description of the uninfected epithelium is based upon both.

Cilia.—Sections of cilia from many different species of animals have been shown to have a characteristic internal structure consisting of 9 peripheral and 2 central fibers (17). In our material, this appearance of cilia has served as a convenient and certain means for differentiating cells of the bronchial epithelium from other types of pulmonary cells. When the distinction between ciliated and non-ciliated cells of the bronchial epithelium was sought, it was found that the plane of the section could include cilia from cells not present in the section and that cells could be cut at such an angle that their cilia were not included. Therefore, for positive evidence that a cell was ciliated, it was necessary to show that cilia extended into the cytoplasm (Figs. 1, 3, 5, 11, 12, 15, 16). When ciliated cells were identified in this manner, it was found that the endoplasmic reticulum in the cytoplasm of non-ciliated cells was so different from that of ciliated cells that a distinction between the two types could also be made on this basis. The differences in endoplasmic reticulum are described below.

Cell Membranes.—The cell walls were seen clearly and showed various degrees of tortuosity (Figs. 1 to 4, 9, 10, 12, 15 to 19). At the ciliated margins of cells, many microvilli protruded from the cell membrane. These structures were present in large numbers among the cilia but were readily distinguished from cilia by their small size and absence of internal fibers (Figs. 1, 3, 5).

Nuclei.—Nuclei were bounded by thin membranes that were sometimes seen to have a double structure. The nucleoplasm was finely granular and one or more nucleoli were usually present (Figs. 2, 4, 6, 7).

Mitochondria.—By staining paraffin sections for study of mitochondria by light microscopy (1), it had been found that cells of the bronchial epithelium contained many rod-like forms. In the thin sections for electron microscopy, mitochondria were usually cut transversely and appeared as round or ovoid bodies of various sizes although occasional oblique cuts resulted in longer forms. The internal structure of mitochondria in the bronchial epithelium was not visualized as readily as in some of the other types of pulmonary cells seen in the same sections. While mitochondria of some septal cells showed very prominent

cristae (18, 19), mitochondria of the bronchial epithelium were usually dense and homogeneous (Figs. 1, 2, 4, 6, 7, 9, 15, 18 to 21). When the period of fixation was increased, density of the mitochondria persisted, but internal cristae were often seen and served as further means of identification (Figs. 3, 10, 13, 22). Lipid granules in the cytoplasm could usually be distinguished from mitochondria by their much greater electron density (Figs. 2, 12) and by the absence of cristae within them.

Endoplasmic Reticulum.—Electron microscopy of the cytoplasm by a number of workers has shown fine structures designated as systems of double membranes (19), lamellae (20), endoplasmic reticulum (21–24), and ergastoplasm (21, 25). Corresponding structures in ciliated cells of the uninfected bronchial epithelium were absent or few in number and when present consisted of scattered small vesicles and short double membraned formations (Figs. 1 to 3). In contrast, the cytoplasm of all non-ciliated cells contained large numbers of small round or ovoid vesicular structures which often appeared to be densely packed together throughout the cytoplasm (Figs. 1, 2, 4, 9, 12, 13). Occasionally, in non-ciliated cells, some of the vesicular forms appeared to be oriented in chains and sometimes long double membranes were present. The striking difference in the amount of endoplasmic reticulum in ciliated and non-ciliated cells served as a valuable means for recognition of the two types of cells and is illustrated in Figs. 1 to 4.

Bronchial Epithelium of Lungs Infected with Influenza Virus

Inclusion Bodies.—In the preceding study by light microscopy (1), inclusion bodies were shown to be present within the cytoplasm of cells in which intact nuclei were demonstrated. The validity of this observation depended to a large extent on the clarity with which nuclei and cell walls could be visualized. In the present work, nuclei and cell membranes of individual cells were distinctive in appearance and could be recognized with certainty so that the presence of intracytoplasmic inclusion bodies was confirmed without difficulty (Figs. 6 to 10, 13 to 15, 18). The location, size, numbers, and shapes of these bodies corresponded very well with the observations made previously except that crescentic forms were not seen. Some of the inclusions were surrounded by empty spaces as if their peripheries had separated from the rest of the cytoplasm (Figs. 10 and 14).

Especial attention was given to the spatial relationships between inclusion bodies and other structures of the cell. It was found that the bodies often occurred in areas of the cytoplasm well separated from nucleus, cell membranes, endoplasmic reticulum, mitochondria, or lipid granules (Fig. 7).

Particles.—Examination of the fine structure of the cytoplasmic inclusion bodies showed the presence of many small particles of fairly uniform size (Figs. 6 to 10, 13 to 15, 18). These particles had a considerably greater electron

density than other cellular constituents but variation in density was occasionally observed (Fig. 18). The particles were often clearly visible as individuals in some parts of a single inclusion while in other areas of the same body were so closely packed together that they could not be recognized singly. Although most inclusions appeared to be composed mainly of small particles, other dense material of round or ovoid form was often found within them (Figs. 6, 9, 10) and occasionally resembled mitochondria (Figs. 9, 10). Sometimes the particles were separated from the rest of the cytoplasm by a membrane (Figs. 9, 13).

Accurate measurements of particle size were not possible because edges of the particles were not sharp and because of some variation from a circular shape. Nevertheless, an effort was made to measure the diameters of a number of the discrete particles and the approximate size was calculated knowing the magnifications due to the instrument and the photographic enlargement. Under these circumstances, diameters were estimated to fall in a range between 50 and 100 mμ and showed general agreement with other methods for determination of the size of Type A influenza virus (26–30).

Linear Formations[10].—The relative sparsity of endoplasmic reticulum in uninfected ciliated cells made it possible to recognize the presence of abnormal linear structures in some ciliated cells of lungs infected with virus. These lines closely resembled forms of endoplasmic reticulum (Figs. 6, 17 to 21). They were usually double and had a twisted appearance (Figs. 6, 18 to 21); they were often found near mitochondria and sometimes appeared to be wrapped around them (Figs. 18 and 19). In some cells, they either appeared to consist of chains of vesicles (Fig. 17) or formed whorls (Figs. 20 and 21). In a few instances, lines of the whorls were in close apposition to each other and resembled foci of forming endoplasmic reticulum (ergastoplasm) described in the pancreas (Fig. 22) (25). Although recognized most readily in ciliated cells, linear structures were also found in non-ciliated cells (Fig. 22). However, in non-ciliated cells, it was difficult to be sure that linear formations were due to viral infection because of the occurrence of similar structures in uninfected non-ciliated cells.

Since the abnormal linear formations resembled the endoplasmic reticulum of other types of cells, and because of the theory that endoplasmic reticulum functions in synthesis of protein, the possibility arose that the linear structures might be involved in the cellular mechanism for viral synthesis. For this reason, observations were made of spatial relationships between abnormal lines and inclusion bodies. No constant relationship could be established. In some instances, the linear structures were near inclusion bodies (Figs. 6, 18)

[10] The designation as linear is used because it describes the actual appearance of these structures in the electron micrographs. The probability that linear formations result from transection of sheets will be discussed below.

but more often they occurred in cells in which inclusions were not seen (Figs. 17, 19 to 22).[11]

Microvilli.—Electron microscopic observations have been reported indicating that influenza virus grows in minute cytoplasmic projections from the surfaces of cells (31–37). In view of these reports, we have compared the microvilli on the ciliated borders of ciliated cells in uninfected and infected bronchi but have not been able to detect any significant difference between them (Figs. 1, 3, 5, 11, 12, 15, and 16).

Because of reports of double membranes of viral particles after osmium fixation of only 20 minutes (38, 39), the possibility arose that a similar structure of influenzal particles on the cellular surfaces might be invisible because

TABLE I

Presence of Inclusion Bodies at Intervals after Exposure to Different Strains of Virus

Strain of virus	Hrs. after inhalation																										
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	23	24	27	40	42	43	45	47
Weiss		0	+*	0	0	0	0	0	0	0	0	0	0	+	+	+		+		+	+		+	+				
Swine	0										0								+									
PR8 (ATCC)‡		0	0	0	0	+	0	+	0	0	+	+	0	+	+		+	+	+									
A¹ (47-2)§																						0				0		+
A¹ (47-3)§																								+	+		0	

* One small indistinct inclusion.

‡ American Type Culture Collection.

§ Unadapted strains.

of the oxidation due to longer fixation (10). For this reason, blocks of lung infected with PR8 strain were fixed for 20 minutes and sections prepared as before. Again, it was not possible to recognize viral particles on the cellular surfaces or to distinguish any alteration in the microvilli (Fig. 16).

Strains of Virus and Times of Observation.—No qualitative difference was noted in the cellular changes produced by the various strains but lesions were more plentiful in lungs infected with the strains of mouse-adapted type A virus. For this reason, efforts to determine the time of earliest cellular changes were made only with 2 of these mouse-adapted strains although times of observation with all the strains are shown in Table I. Unadapted strains were included in the study because of their property of multiplication in the lung without the production of macroscopic lesions (40, 41). If the inclusion bodies were microcolonies of virus, it was thought that they should be present after inhalation of unadapted strains of virus and this was found to be the case (Fig. 14).

Sections were made at hourly intervals after inhalation of the Weiss and PR8 strains of virus in order to detect the earliest lesion due to viral action

[11] Our initial finding that abnormal endoplasmic reticulum preceded the appearance of inclusion bodies (6) could not be conclusively confirmed in further observations.

and, if possible, to recognize viral precursors. It was found that definite inclusion bodies were present as early as 13 hours after inhalation of the Weiss strain and 5 hours after the PR8 strain, but no definite evidence concerning viral precursors could be obtained. Many round and ovoid forms of endoplasmic reticulum were seen and their resemblance to "doughnut" forms (42–44) was noted, but their evolution into possible viral particles could not be demonstrated because they were present at all periods after inhalation of virus and were also present in uninfected cells.

DISCUSSION

Cytoplasmic inclusion bodies of the bronchial epithelium infected with influenza virus have been shown in this report to consist mainly of particles of a size to be expected if they were particles of the virus itself. This evidence supports the theory that the inclusion bodies are primarily microcolonies of viral units, since it seems unlikely that non-viral particles of this size would form in degenerative cytoplasmic lesions resulting from viral action. Evidence already exists to show that intracellular inclusion bodies caused by some viral infections contain the infectious units.

While the presence of inclusion bodies within cells does not necessarily indicate the action of a virus (45), it is well established that many viruses induce the formation of cytoplasmic or nuclear inclusions (46, 47). In many viral infections, the inclusion bodies are believed to consist of intracellular colonies of the infectious agents with or without a matrix derived from the cell, but in other instances viral inclusions are thought to be products of degeneration in damaged cells.

With the large viruses such as psittacosis, lymphogranuloma venereum, vaccinia, fowl pox, and molluscum contagiosum, the observations of many workers using methods of light microscopy indicate that the cytoplasmic inclusions contain particles that can be identified almost certainly as elements of the infectious agents (46-48). The use of electron microscopy for examination of the cytoplasmic inclusions of these large viruses has usually, but not always, shown the presence of virus-like particles within them. With the earlier techniques of electron microscopy, virus-like particles were seen in the inclusion bodies of ectromelia (49), vaccinia (50, 51), molluscum contagiosum (52), and probably fowl pox (53), but recent work with unshadowed sections after osmium fixation appears to give better results and viral particles can be identified with greater certainty. Under these conditions, the inclusion bodies of molluscum contagiosum showed virus-like particles (44). Also, tissues infected with vaccinia and ectromelia revealed probable viral particles within dense cytoplasmic inclusions or less dense matrices, and in other portions of the cytoplasm (44). However, in some instances, dense cytoplasmic inclusions were found that did not contain any virus-like particles. In another study by a similar technique (39), probable viral particles of vaccinia and fowl pox were found in the cytoplasm of infected cells, and in the case of fowl pox association of granular material with particles was thought to correspond to the inclusions of light microscopy. The inclusions however could not be identified as such by electron microscopy.

In addition to the large viruses noted above, cytoplasmic inclusion bodies of plant and insect viruses have received considerable study by electron microscopy and probable viral particles have been found within them (54). However, electron micrographs of Negri bodies in rabies did not show viral particles (55, 56), and the virus-like particles of infectious myxomatosis were apparently not associated with inclusion bodies (57). In the pancreatic lesion of Coxsackie virus, a few dense cytoplasmic inclusions were found that did not contain viral particles but some clumps of virus-like particles were seen elsewhere in the cytoplasm (58).

In the case of viral inclusions within the nucleus, virus-like particles are usually not believed to be visible by light microscopy (46). In early studies with the electron microscope, neither viral particles nor inclusions were found in nuclei of cells infected with herpes virus (59) but later shadow-cast sections showed nuclear inclusions containing virus-like particles (60). In recent work with unshadowed osmium-fixed sections, particles of herpes virus have been identified with more certainty and have been found both in the nucleus and cytoplasm (38). Use of a similar technique with a virus related to herpes, showed virus-like particles in the nucleus (61). Electron micrographs of nuclear inclusions in some plant and insect viral diseases have also shown probable viral particles (54).

In addition to microscopic observation of particles, evidence for the presence of virus in cytoplasmic and nuclear inclusion bodies has been obtained by microdissection (62-65), differential centrifugation (66), and the use of fluorescent antibody (67, 68).

In view of the finding that particles resembling influenza virus are present in cytoplasmic inclusions in the bronchial epithelium of mice, it is necessary to note that use of fluorescent antibody for intracellular localization of influenza virus in embryonated eggs (69) and in nasal epithelium of ferrets (70) has shown viral antigen diffusely distributed both in the nucleus and cytoplasm. Reasons for such apparent variation in localization of virus within cells cannot be given at the present stage of knowledge but may be due to host factors inherent in each particular type of cell.

The appearance of microvilli on ciliated cells of the bronchial epithelium is quite similar to that of those noted on other ciliated epithelia (17). However, the presence of microvilli is significant not only because they occur on ciliated and other types of cells (22, 25, 71, 72), but also because of the theory that influenza virus grows in filaments on the surfaces of cells (31–37).[12] In this

[12] Most of the indications that influenza virus may grow on the surfaces of cells have come from studies of tissue cultures or chick embryo membranes. However, electron microscopic observations of sectioned pulmonary tissue of mice infected with influenza virus have shown filamentous forms apparently growing on the surfaces of alveolar walls (32). In the report cited, it may be noted not only that the technique differed considerably from that of the present work but also that sections were prepared at a much longer interval after exposure to virus. A recent abstract concerning electron microscopy of the lungs of mice infected with influenza virus indicates that viral particles could be found in the bronchioles and that a "peculiar swelling of cilia was observed" (73).

connection, it appears that the interpretation of influenza virus growing on the surface depends upon the visual distinction between viral filaments and normal or altered microvilli of cell surfaces. In our material, we have been unable to see any difference between the microvilli on epithelial cells of control and infected bronchi. Therefore we cannot say that our observations give any support to the assumption that the virus grows on cellular surfaces.

The abnormal linear formations seen most clearly in ciliated cells were associated with the viral infection, and the possibility arises that these lines consist of filamentous forms of influenza virus (74–78). We believe, however, that these structures are abnormal forms of endoplasmic reticulum that have appeared in response to infection with the virus. It seems unlikely that the lines consist of fibers or filaments because linear formations seen in thin sections must be due to the transection of planes or sheets of material. We agree with the interpretation of others (19, 24, 25) that the thinness of these sections is such that the probability of cutting thin filaments longitudinally is very small. Filaments would be expected to have the appearance of small dots.

The possible relationship of endoplasmic reticulum to the synthesis of protein (21, 25) may conceivably have a bearing on the synthesis of influenza virus. Although inclusion bodies were found to occur in areas of cytoplasm devoid of endoplasmic reticulum, it is possible that the functional effect of endoplasmic reticulum might be exerted at some distance within the cytoplasm. Also, since cytoplasmic constituents are in motion during life (79), the fixation of some cells might occur at a time when viral colonies and endoplasmic reticulum were temporarily separated.[13] Similar reasoning might apply in the case of inclusions occurring in areas of the cytoplasm remote from the nucleus or lacking in mitochondria.

SUMMARY

Recently improved methods for visualization of thin tissue sections by electron microscopy have been applied to the study of early changes in the bronchial epithelium of mice infected by inhalation of aerosols of influenza virus.

In confirmation of previous findings by the authors, inclusion bodies have been demonstrated in ciliated and non-ciliated cells of infected bronchial epithelium. In addition to 3 strains of mouse-adapted Type A virus, 2 unadapted strains gave qualitatively the same results. The inclusion bodies were found to be composed largely of particles of a size estimated to correspond to the known size of influenza virus. The viral lesion of the cytoplasm was also associated with linear formations which were thought to be abnormal forms of endoplasmic reticulum. Well developed microvilli were found on the ciliated

[13] Suggested by Dr. Igor Tamm.

borders of ciliated cells, but no evidence was found of viral growth in this region.

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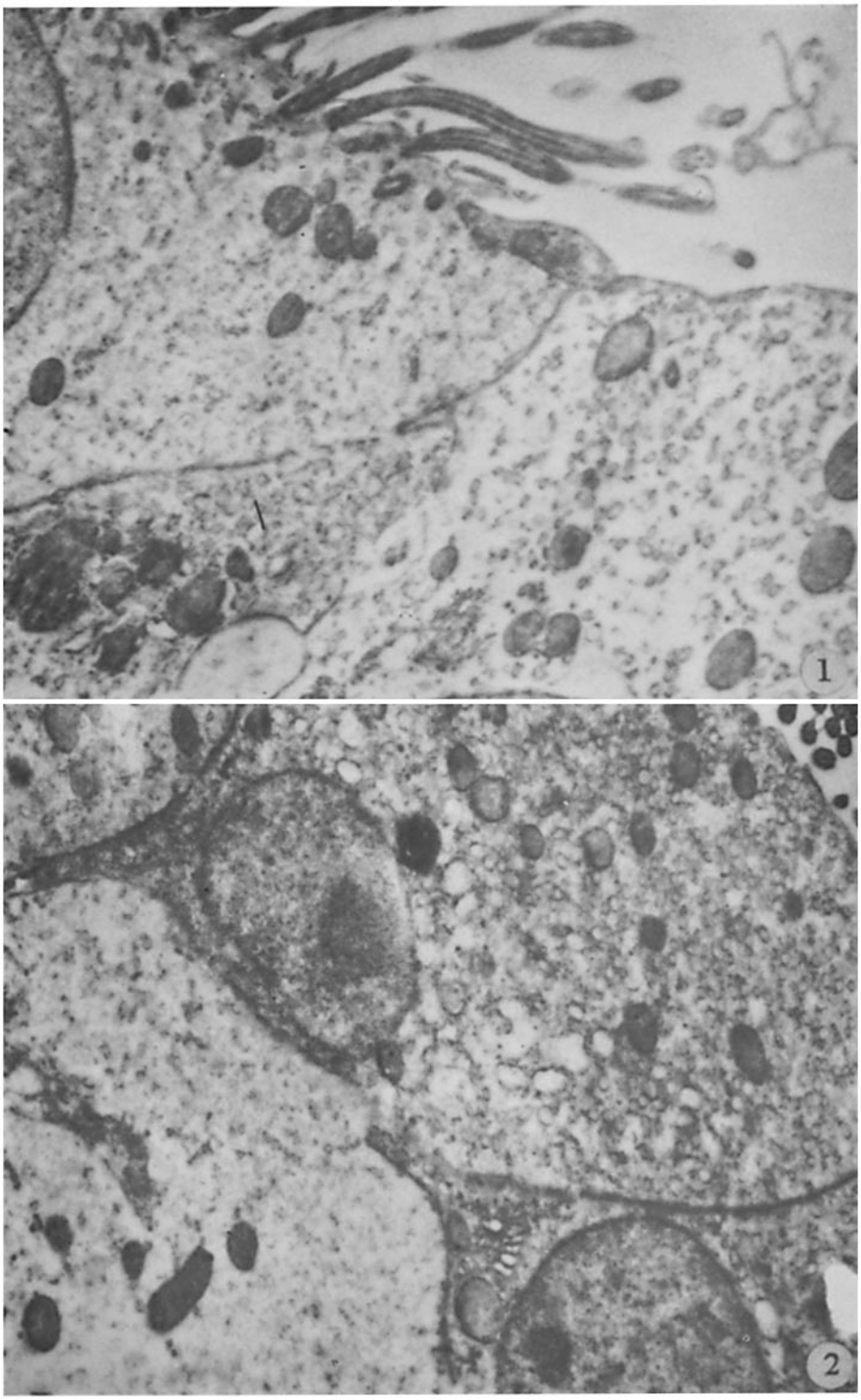
EXPLANATION OF PLATES

PLATE 34

FIG. 1. Ciliated and non-ciliated cells of the normal bronchial epithelium. A portion of the nucleus of the ciliated cell is present in the upper left hand corner, and the cytoplasm of this cell is nearly devoid of endoplasmic reticulum. Microvilli are present among the cilia. In the lower right hand corner is a portion of the cytoplasm of a non-ciliated cell and numerous round and ovoid forms of endoplasmic reticulum are shown. Fixation 24 hours. × 15,000.

FIG. 2. A portion of the cytoplasm of a normal ciliated cell is shown in the lower left-hand corner and is seen to have only a few elements of endoplasmic reticulum. The cytoplasm of the normal non-ciliated cell in the upper right hand corner appears nearly packed with round and ovoid forms of endoplasmic reticulum. Close to the nucleus of the non-ciliated cell is a dense granule (probably lipid) easily distinguished from the mitochondria. Fixation 6 hours. × 15,000.

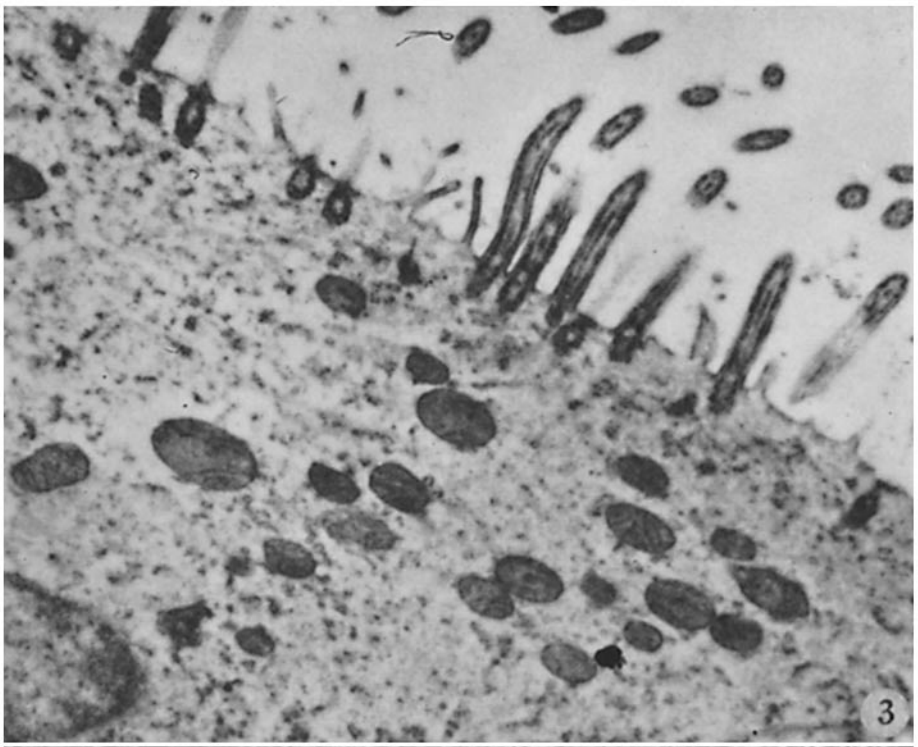


(Harford *et al.*: Cellular changes due to influenza virus)

PLATE 35

FIG. 3. Normal ciliated cell with cilia extending into the cytoplasm, as well as some microvilli cut longitudinally. The cytoplasm shows only a few elements of endoplasmic reticulum. Cristae can be seen in the mitochondria. Fixation 18 hours. × 15,000.

FIG. 4. Portions of 3 normal non-ciliated cells with numerous round and ovoid forms of endoplasmic reticulum. The cell on the right shows a normal nucleus with nucleolus. Fixation 18 hours. × 15,000.



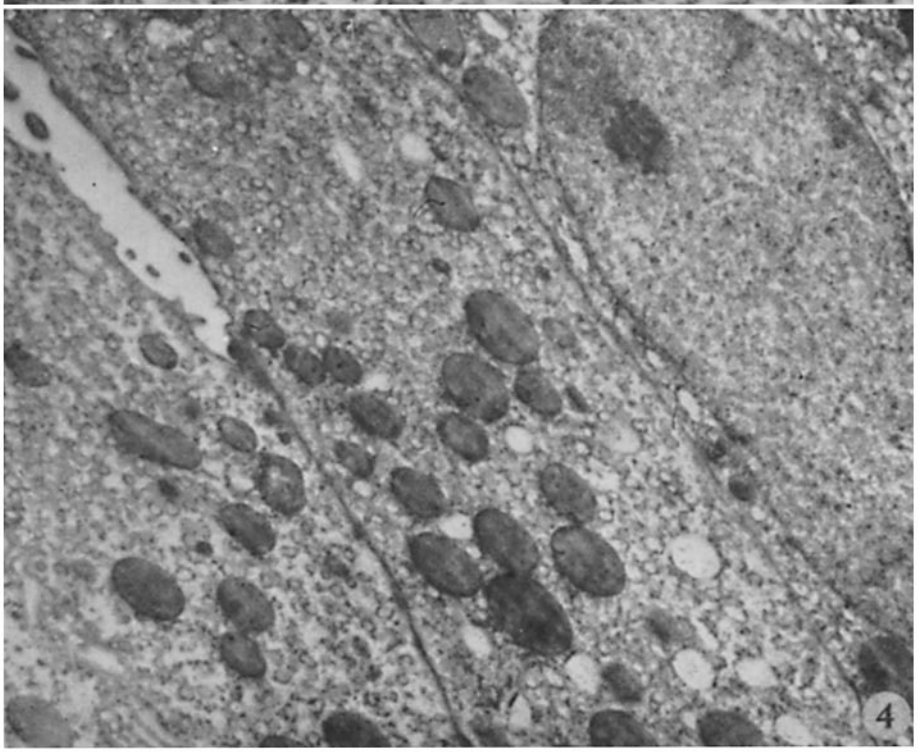


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PLATE 36

FIG. 5. Margins of ciliated cells 14 hours after inhalation of heat-inactivated virus. Many cilia and microvilli are cut transversely. Fixation 24 hours. × 20,000.

FIG. 6. Cells of the bronchial epithelium 15 hours after inhalation of virus. The cytoplasm of the upper cell contains an inclusion body to the right of a normally appearing nucleus. Particles and a large ovoid object are visible within the inclusion. The cytoplasm also contains some dense mitochondria and double linear structures taken to be abnormal forms of endoplasmic reticulum. In the lower cell, the cytoplasmic inclusion body contains many small particles and is also to the right of the nucleus. In the lower left hand corner, a segment of a nucleus and more linear formations are seen. Weiss strain. Fixation 4 hours. Low magnification.

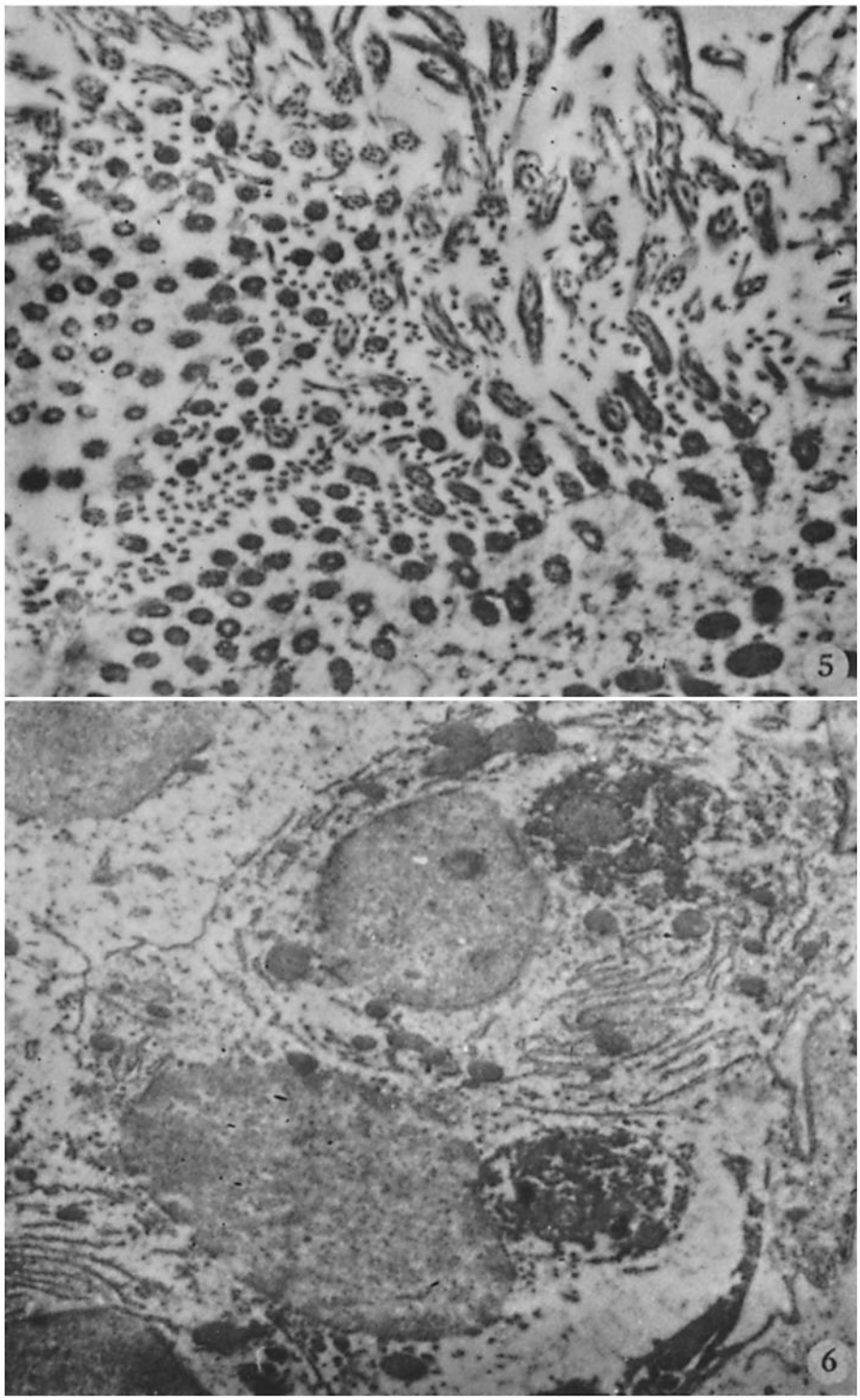

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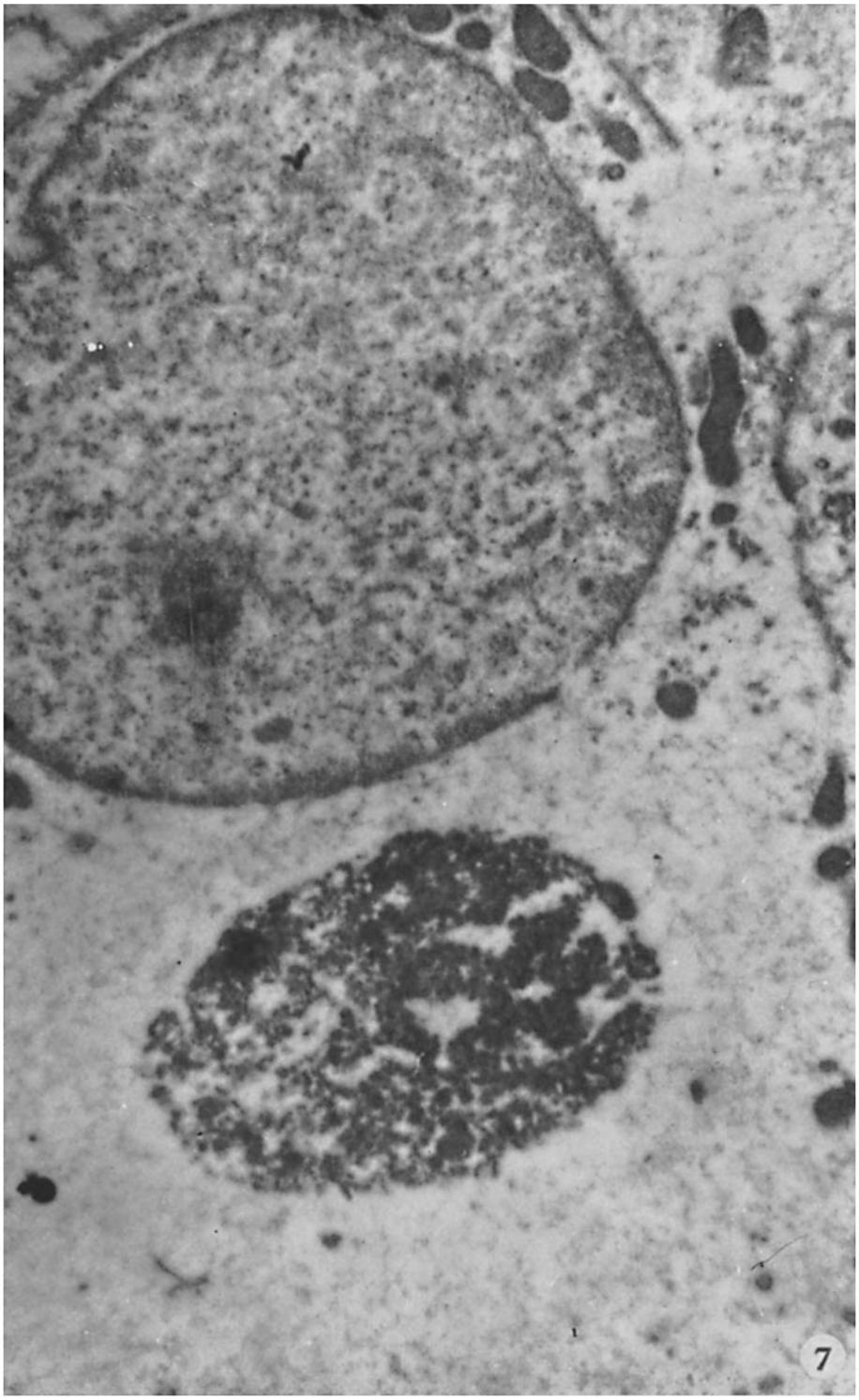
PLATE 37

FIG. 7. A large inclusion body in the cytoplasm of a ciliated cell 23 hours after inhalation of virus. The inclusion body contains many electron-dense virus-like particles and is located in an area of cytoplasm devoid of other cytoplasmic organelles. The nucleus contains a nucleolus and shows no lesion. Weiss strain. Fixation 4 hours. × 15,000.



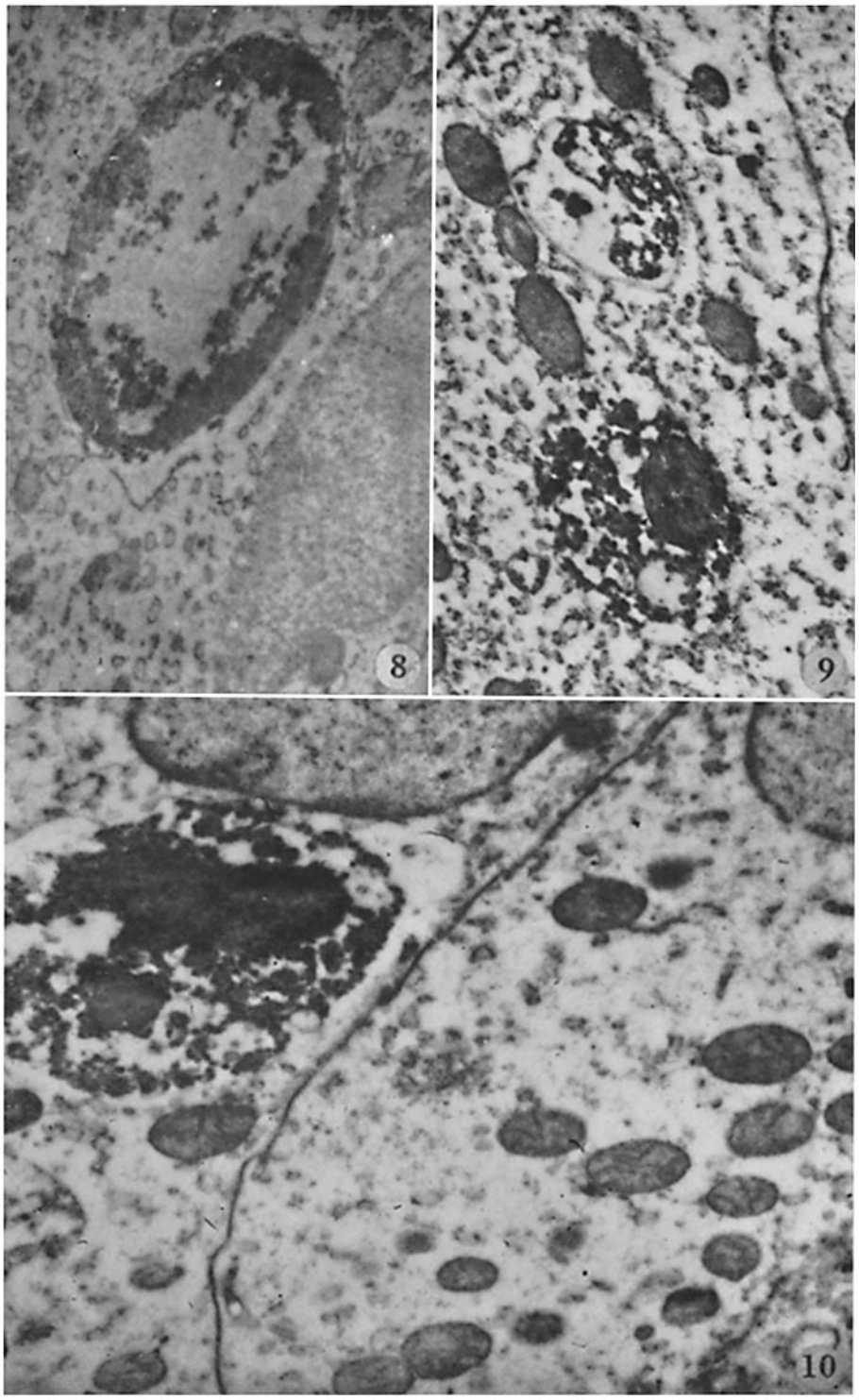
(Harford *et al.*: Cellular changes due to influenza virus)

PLATE 38

FIG. 8. A large inclusion body in the cytoplasm of a non-ciliated cell 15 hours after inhalation of virus. The sparsity of particles in the center of this inclusion makes it easier to distinguish them as individuals. The numerous round and ovoid forms of endoplasmic reticulum appear normal as do the nucleus and mitochondria. Weiss strain. Fixation 4 hours. × 20,000.

FIG. 9. Portion of the cytoplasm of a non-ciliated cell 14 hours after inhalation of virus, showing two inclusion bodies containing particles. The upper inclusion appears to be surrounded by a membrane and the lower one contains a large dense ovoid object, possibly a mitochondrion. This object can be compared with normally appearing mitochondria in the rest of the cytoplasm. The small, ovoid forms of endoplasmic reticulum appear normal. PR8 strain. Fixation 24 hours. × 25,000.

FIG. 10. A large inclusion body in the cytoplasm 18 hours after inhalation of virus. In addition to particles, this inclusion contains an object similar in size, shape, and general appearance to a mitochondrion. This object does not contain cristae but resembles a mitochondrion surrounded by viral particles. PR8 strain. Fixation 24 hours. × 30,000.



(Harford *et al.*: Cellular changes due to influenza virus)

PLATE 39

FIG. 11. Ciliated borders of cells 6 hours after inhalation of virus. Numerous, normally appearing microvilli are present among the cilia. Weiss strain. Fixation 4 hours. × 15,000.

FIG. 12. Portions of ciliated and non-ciliated cells 9 hours after inhalation of virus. Cilia and apparently normal microvilli are shown. Some dense granules (probably lipid) are readily distinguished from mitochondria. PR8 strain. Fixation 24 hours. × 15,000.

FIG. 13. A small (probably early) inclusion in a non-ciliated cell 18 hours after inhalation of virus. One of the particles in this inclusion is surrounded by a membrane and is mentioned only to indicate that it is not a typical finding. The inclusion itself is also surrounded by a membrane. Endoplasmic reticulum and nucleus appear normal. Some of the mitochondria show cristae and some do not. PR8 strain. Fixation 24 hours. × 20,000.

FIG. 14. A large inclusion body composed mainly of particles, in the cytoplasm of a ciliated cell 42 hours after inhalation of unadapted virus. Some separation of the inclusion body from the other cytoplasmic constituents has taken place. 47-2A prime strain. Fixation 24 hours. × 15,000.

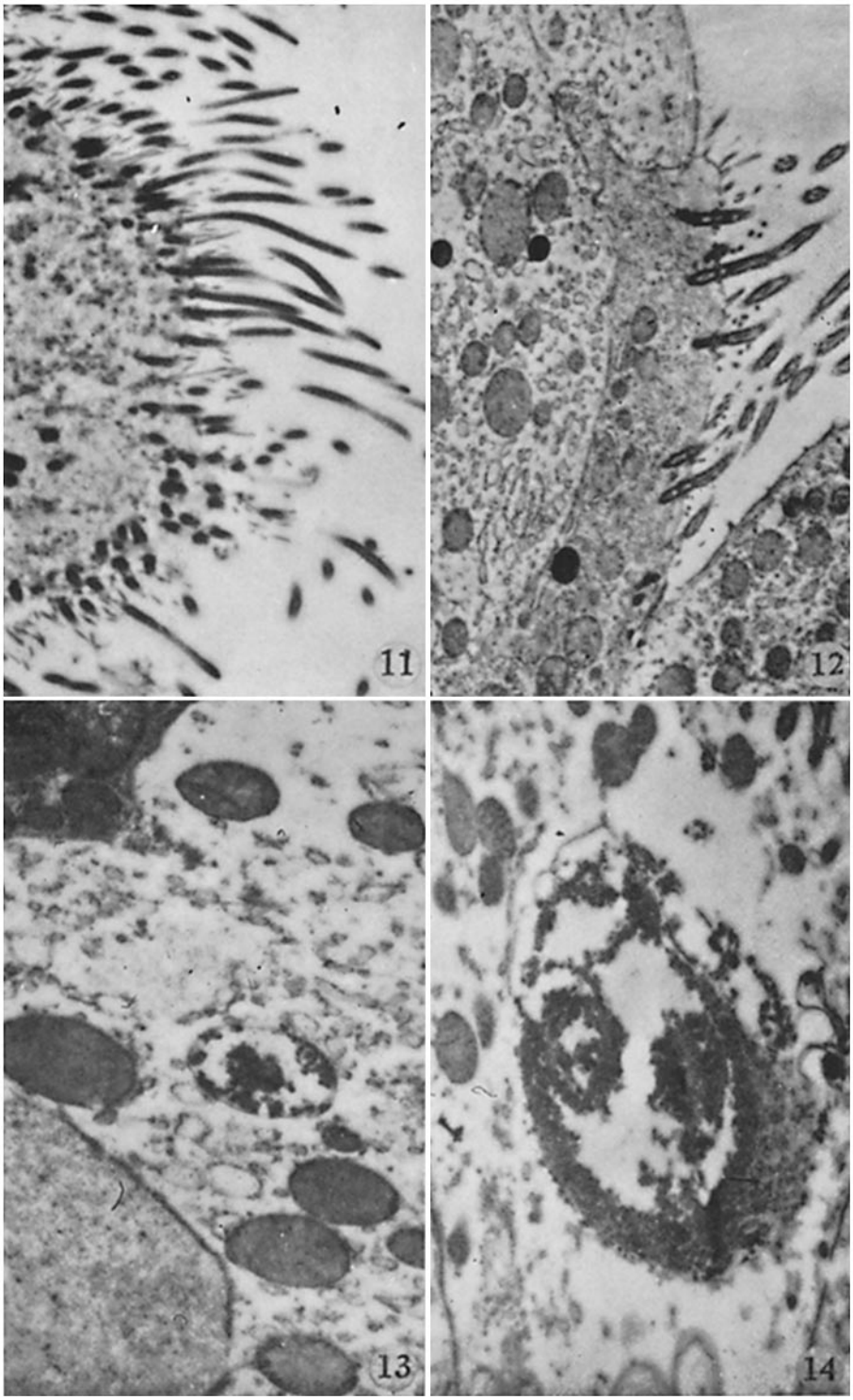


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PLATE 40

FIG. 15. Cilia with apparently normal microvilli on cells 23 hours after inhalation of virus. The upper cell contains an inclusion body with particles. Weiss strain. Fixation 4 hours. × 10,000.

FIG. 16. Apparently normal microvilli of cells 18 hours after inhalation of virus. The microvilli are in close apposition and appear as fine parallel lines in the center of the field. Sections of cilia are shown above. Cytoplasm of cells on both sides contained inclusion bodies (not shown). PR8 strain. Fixation 20 minutes. × 20,000.

FIG. 17. Section through the cytoplasm of two cells 27 hours after inhalation of virus. In the upper cell, curved chains of small vesicular structures are shown and these are interpreted as abnormal forms of endoplasmic reticulum due to viral infection. The cytoplasm elsewhere in this cell is lacking in the round or ovoid forms of endoplasmic reticulum and hence this cell is believed to have been ciliated. In the lower cell, the rootlets of cilia are shown at the left pole and endoplasmic reticulum is absent. A few small, dense granules between the two cells may be extracellular viral particles. Weiss strain. Fixation 4 hours. × 10,000.

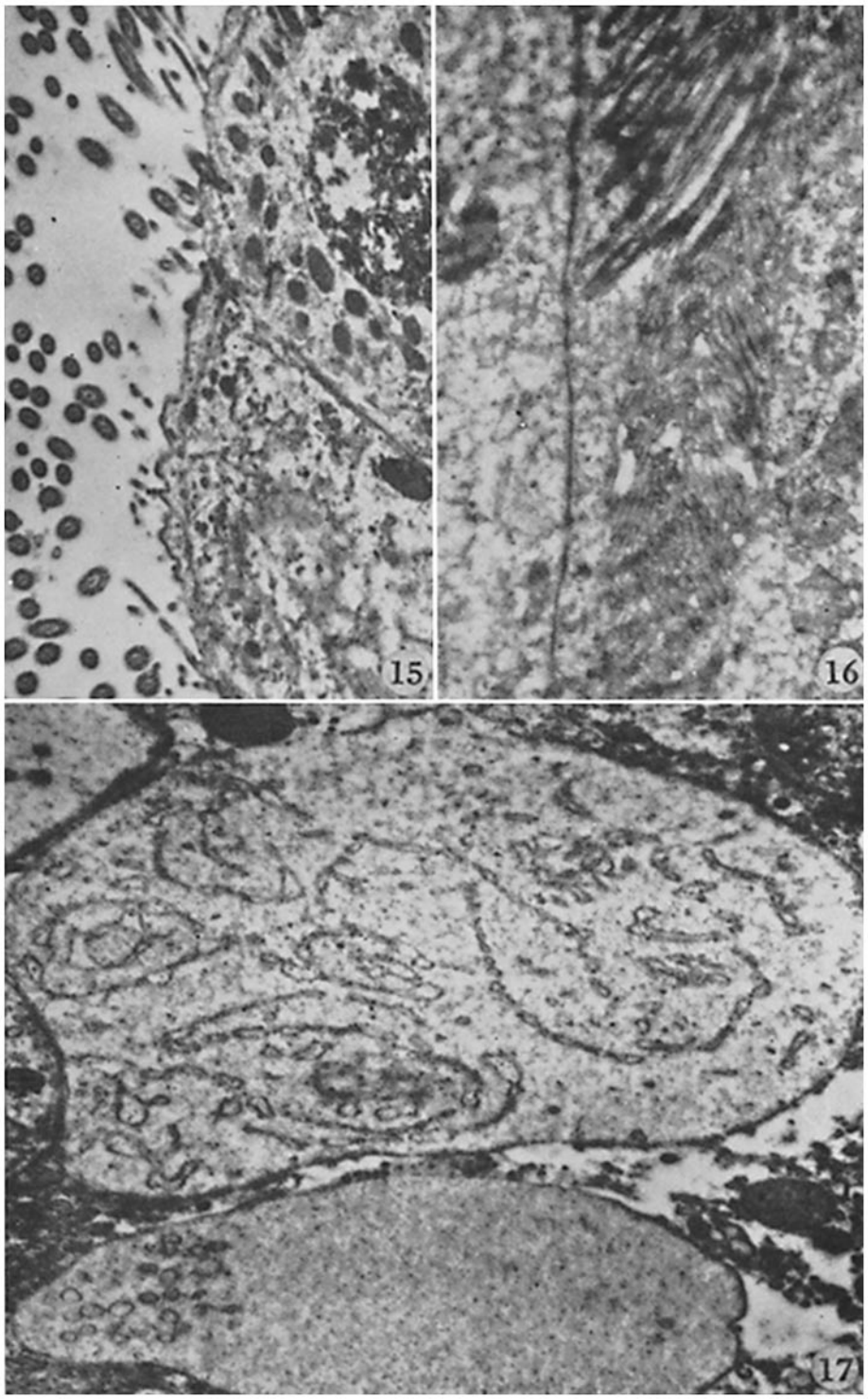

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PLATE 41

FIG. 18. Curved linear formations in a cell 23 hours after inhalation of virus. Lines are often double and surround the nucleus and mitochondria. These lines are interpreted as abnormal forms of endoplasmic reticulum due to viral infection. Two inclusion bodies contain particles of varying density. The cytoplasm does not contain the round and ovoid forms of endoplasmic reticulum characteristic of non-ciliated cells. The mitochondria and nucleus appear normal. Cytoplasm of another cell (shown at left) contains an inclusion body. Dense particles in upper left hand corner may be extracellular virus. Weiss strain. Fixation 4 hours. × 10,000.

FIG. 19. Portion of the cytoplasm of a cell 15 hours after inhalation of virus. Linear formations (probably abnormal endoplasmic reticulum) in close apposition to mitochondria. Weiss strain. Fixation 4 hours. × 20,000.

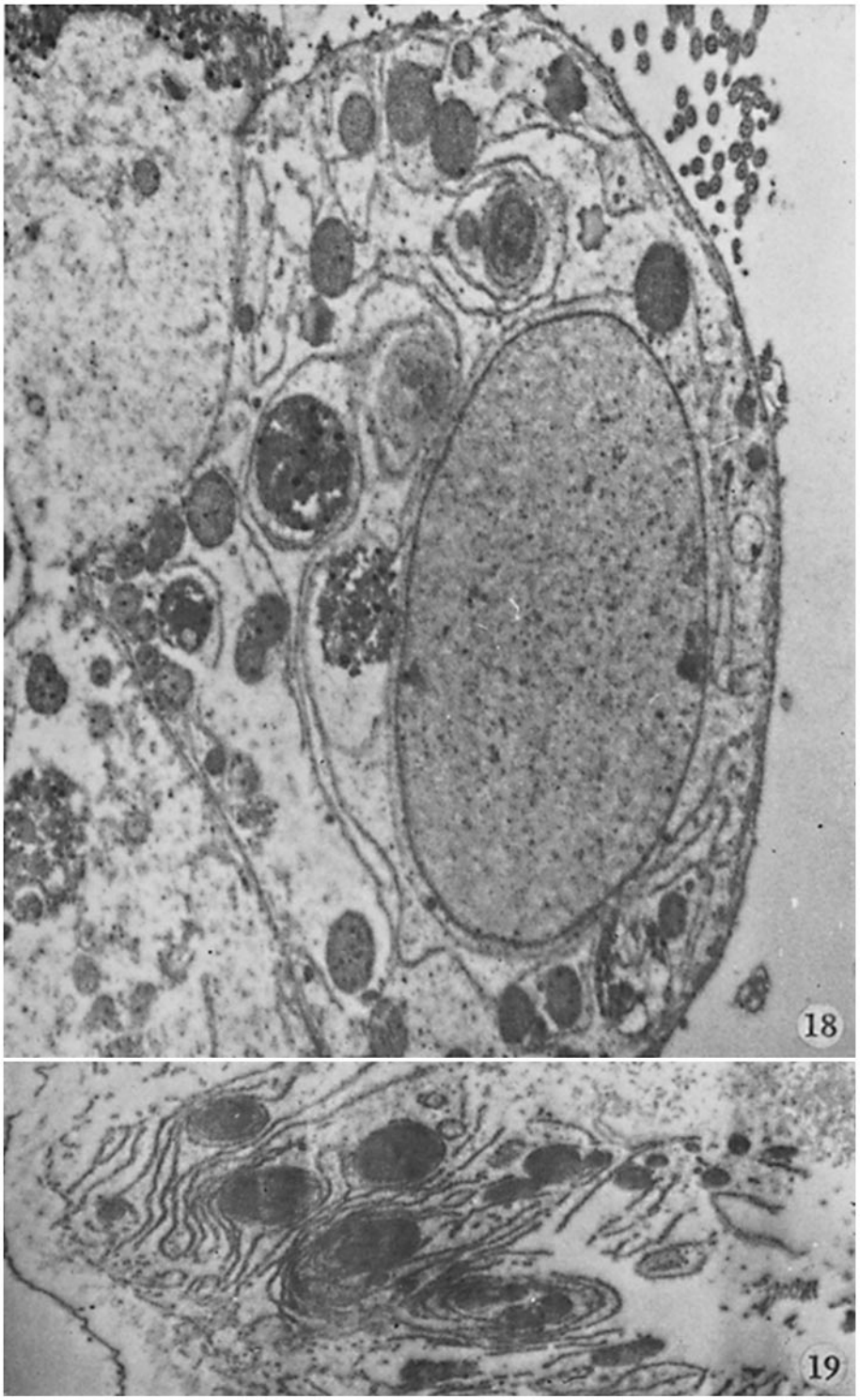

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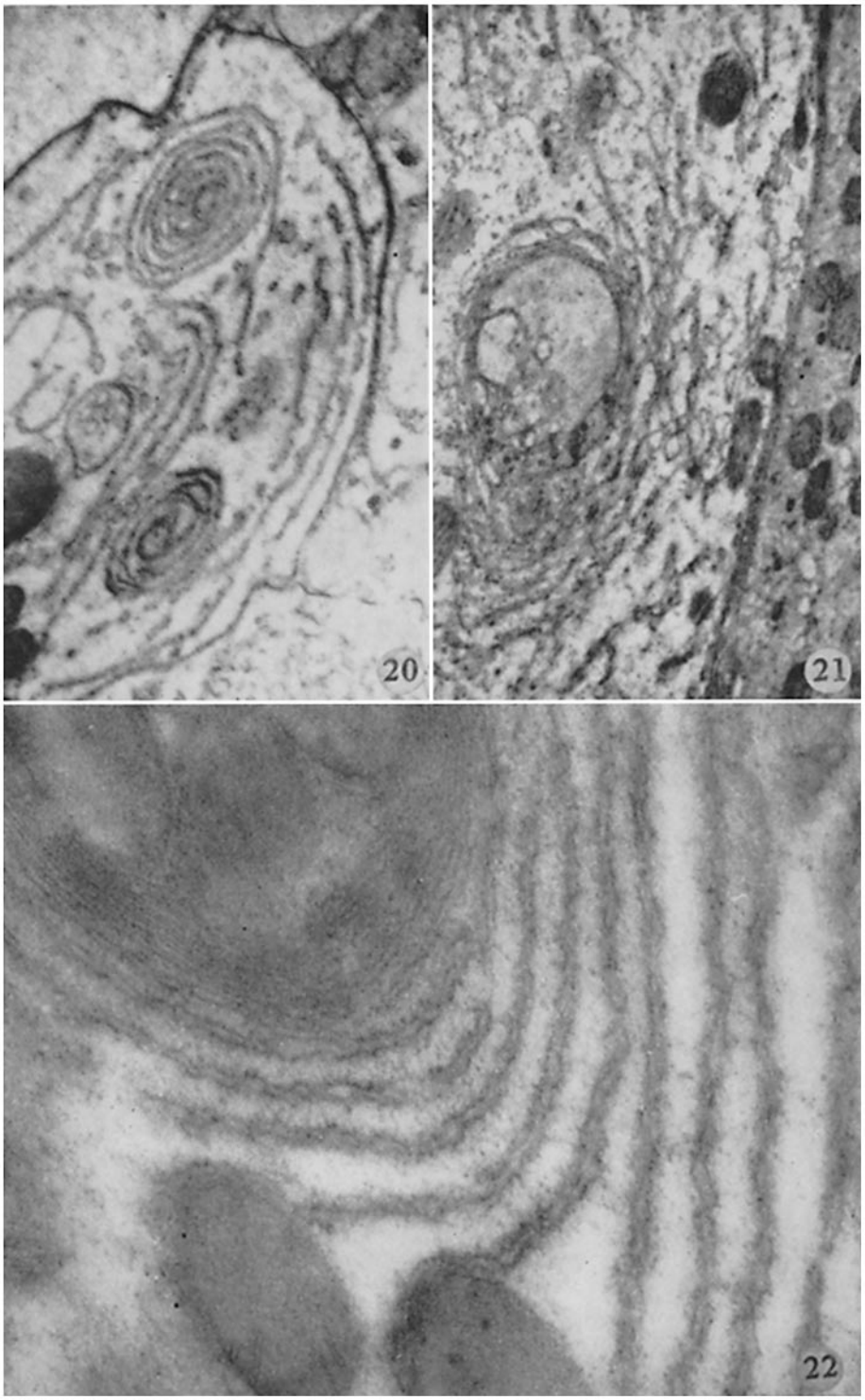
PLATE 42

FIG. 20. Cytoplasm of a cell 27 hours after inhalation of virus. Whorls of double lines surround small vesicles and probably consist of abnormal endoplasmic reticulum. The forms of normal endoplasmic reticulum of non-ciliated cells are so few in number that this was probably a ciliated cell. Weiss strain. Fixation 4 hours. × 10,000.

FIG. 21. Cytoplasm of a ciliated cell 27 hours after inhalation of virus. Whorls of wavy lines surround vesicular structures and are thought to be abnormal endoplasmic reticulum. Weiss strain. Fixation 4 hours. × 10,000.

FIG. 22. Portion of the cytoplasm of a non-ciliated cell 5 hours after inhalation of virus. The curved double lines probably consist of endoplasmic reticulum due to viral infection. This relationship to viral infection is not certain, however, because of the occasional occurrence of similar double lines in uninfected non-ciliated cells. Numerous round and ovoid forms of normally appearing endoplasmic reticulum were present in other portions of the cytoplasm (not shown) and permitted the identification of this cell as non-ciliated. In the upper part of the picture is a whorl of lines in close apposition to each other. This structure resembled closely foci of forming endoplasmic reticulum (ergastoplasm) (25). The ovoid structures within the whorl resemble the definite mitochondria in the lower fart of the picture. PR8 strain. Fixation 24 hours. × 40,000.



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