ELECTRON MICROSCOPY OF HELA CELLS INFECTED WITH ADENOVIRUSES*, ‡

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Present techniques for electron microscopy of tissue sections have provided opportunities for study of relationships between viruses and structural components of the cells they infect. The object of the present investigation was to use such methods for examination of the newly discovered adenoviruses, some of which have been shown to cause a significant amount of clinically recognized human disease.[1] It was hoped that viral particles might be recognized within the cell, and to gain knowledge from their location concerning the intracellular environment in which multiplication takes place. In addition, it was thought that examination of the viral lesion might give information concerning the nature of viral injury to cellular structures.[2]

Methods and Materials

HeLa Cells.—All the experiments were done with the HeLa strain of human carcinoma cells (16, 17) maintained in tissue culture. Techniques were those of Syverton, Scherer, and Elwood (18) except for minor modifications. Cells for transfer were scraped from the glass (3). The first experiments utilized tube cultures and fluids obtained from Microbiological Associates, Inc.,[3] the remaining experiments were done with cells grown in bottles in our laboratory and distributed to tubes or slides as needed. The additional tissue culture fluids were maintenance solutions containing tryptose phosphate (19) or yeast extract and glucose (20), and a nutrient solution consisting of equal parts of human serum, balanced salt solution, and tryptose phosphate.

Viruses.—Two strains of virus were kindly furnished by Dr. Harold Ginsberg. One strain

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[1] Names for these viruses include APC (adenoidal-pharyngeal-conjunctival) (1), RI (respiratory infection) (2), and ARD (acute respiratory disease) (3). Further references are given in these and other papers (4–13).

[2] Preliminary reports of the findings have been made (14, 15).

[3] Washington, D. C.

designated ARD (3) had been isolated originally as RI-67 (21) and will be called type 4. The other strain was designated AD No. 3 (22) and will be called type 3.

Two strains of virus were kindly furnished by Dr. R. J. Huebner as prototype strains of APC agents and were designated as follows:—

"Type 1 (pool 106) 6th passage fluid 'Adenoid 71'; isolated October, 1953, by inoculation of HeLa cell cultures with a suspension of adenoid tissue from a 5 year old girl.

Type 2 (pool 38) 15th HeLa passage fluids 'Adenoid 6'; isolated from spontaneously degenerating tissue cultures of adenoids from 7 year old girl, March, 1953."

The infectious inoculum for each culture tube to be examined by electron microscopy consisted of 0.1 ml. of undiluted fluid prepared as follows: Cells grown in bottles were washed twice with balanced salt solution and covered with maintenance solution containing virus. 48 hours later when the cytopathogenic effect had taken place and some cells had come off the glass, the bottles were subjected to 6 cycles of freezing and thawing to disrupt the cells. After removal of large particles by centrifugation, the supernatant fluid was dispensed in small amounts and stored in a dry ice box until use.

Preparation of Cells for Electron Microscopy.—Methods for cutting thin sections and use of the electron microscope were essentially the same as used in previous work (23). The fixative in most of the experiments consisted of 1 per cent osmium tetroxide buffered to pH 7.4–7.5 (Palade (24)). For observation of cytoplasmic structures, a similar fixative containing dichromate and osmium tetroxide was also used (Dalton (25)).

Since HeLa cells adhere closely to the glass of the tube during growth, some trials were needed to determine satisfactory techniques for bringing the fixative into contact with the cells. At first, knowing that the function of extracellular cement depends upon ionized calcium (26), we removed the cells from the glass by incubation for 30 minutes in a 4 per cent solution of sodium citrate. The cells were collected by low speed centrifugation and subjected to fixation, washing, dehydration, and embedding by similar centrifugation. In the final embedding, gelatin capsules were maintained in an upright position by insertion into holes in a brass cylinder the size of a centrifuge cup.

Blocks prepared in this manner usually contained gritty material that damaged the edges of glass knives. Therefore, except in later infections when the cells came off the glass spontaneously, the technique was changed as follows: The cells were allowed to remain on the glass surfaces of the tubes and were carried in situ through the processes of fixation, dehydration, and preliminary infiltration. Duration of fixation with Palade's fluid was 30 minutes and with Dalton's solution 1 or 5 minutes. Each subsequent solution was applied for 5 minutes. For final embedding in the capsules, the cells were detached gently from the glass with a small metal spatula under a dissecting microscope and transferred to the gelatin capsules by capillary pipette. Sedimentation of cells in the capsules took place by gravity.

Latex Spheres for Estimation of Particle Size.—Although size of structures in electron micrographs of tissue sections can be estimated by knowing the amounts of microscopic and photographic magnification, determination of the size of virus-like particles in our sections was made by use of latex spheres in a manner similar to that employed for determination of particle size of purified preparations of other viruses (27, 28). A suspension of polystyrene spheres[4] was diluted 1:20 with 2 per cent sodium acetate and converted into an aerosol with a glass nebulizer (De Vilbiss No. 40) under an air pressure of approximately 330 mm. of mercury. Sections were allowed to dry on grids in the usual manner and were placed in a glass bottle connected with the nebulizer. After exposure to droplets for about 30 minutes, the spheres were distributed fairly uniformly over the sections and were present

[4] Particle diameter 0.340 μ (standard deviation 0.005 μ). Run no. 15 N-7. Generously supplied by the Dow Chemical Co.

singly or in small clumps. Measurements were made of virus-like particles and latex spheres occurring in the same fields by examining the negatives under a dissecting microscope with a micrometer scale in one ocular.

Light Microscopy.—Cells were grown directly on slides by the following technique: Individual slides were held horizontally in shallow staining dishes and were flooded to a depth of approximately 2 mm. with suspensions of cells in nutrient fluid prepared as by the usual technique for transplantation. The top of each staining dish was closed by placing another inverted dish upon it, and the edges were sealed with cellophane tape or parafilm. Cells grew on the surfaces of such slides after incubation for 24 hours.

The cells were stained by the Feulgen method (29) using orange G as a counterstain (30). Stains were also carried out for lipide by oil red O (31) and for mitochondria (31).

RESULTS

Virus-Like Particles in the Nucleus.—Some nuclei of cells from infected cultures showed clusters of particles with a distinctive appearance (Figs. 1, 3 to 13). The particles were small, uniform in size, and round or ovoid in shape. Occasional particles were found to have enveloping membranes and internal bodies (Figs. 11 to 13). Arrangement of particles within the clusters was commonly found to simulate crystal formation (Figs. 1, 5, 7 to 9), but numerous clumps of particles were seen without regular positions. Crystal-like arrangement was uncommon with types 1 and 2, and the particles associated with these strains usually appeared to have large spaces between them (Figs. 6, 7, 10).

Since all the cells in a tube were intimately exposed to the viral inoculum in the maintenance solution, it was thought that every cell would be infected. It was surprising to find, therefore, that only a minority of cells (roughly estimated at 5 to 25 per cent) revealed virus-like particles in their nuclei.

One possibility for explanation of this phenomenon was that some cells in non-nutrient maintenance solution might have deteriorated to such an extent that they were no longer capable of supporting viral growth.

In order to investigate this possibility, freshly grown cells were inoculated with type 3 virus in maintenance solution and incubated for 2½ hours. At the end of this time, the maintenance solution and virus were replaced with nutrient solution containing only 10 per cent human serum (32) and incubation continued for an additional 24 hours. By this procedure, antibody in the human serum would not be expected to inhibit viral growth and yet the human serum would assist in furnishing optimal conditions for growth and susceptibility of cells. It was found that the viral cytopathogenic effect took place under these conditions and that cells without virus in control tubes grew well. Nevertheless, electron microscopy of cells with cytopathogenic effect did not show an increase in proportion of nuclei containing virus-like particles.

Estimation of Particle Size.—Latex spheres occurring in the same fields with virus-like particles are shown in Figs. 5 and 8. The largest particles were chosen for measurement so as to avoid low values in those instances in which the plane of the section failed to pass through the center of the particle. Some particles

had an ovoid shape, possibly due to compression by the knife (33), and in these cases the longer dimensions of the particles were measured. It was found that 119 particles in cells infected with type 4 virus had mean measurements of 67 mμ (S.D. ± 9), and 103 particles in cells infected with type 3 virus had mean measurements of 64 mμ (S.D. ± 10).

Lesions of the Nucleus.—Irregular masses of amorphous material within the nucleoplasm were found to occur in close apposition with virus-like particles (Figs. 1, 3 to 5, 7, 8, 11, 12), and sometimes the particles appeared to be embedded within the masses (Fig. 1, 5, 8). Nucleoli were also frequently in apparent contact with virus-like particles. In nuclei with virus-like particles, the nuclear membranes occasionally contained structures resembling targets, that is, single granules located within broad portions of nuclear membrane were surrounded by round clear areas (Fig. 4). Large vacuoles with indistinct margins were usually present in the nuclei of cells infected with type 2 virus (Fig. 7). Nuclei without virus-like particles from cells of infected cultures did not show any change distinguishing them from nuclei of uninfected cultures.

Although virus-like particles were present 24 to 26 hours after infection, the general architecture of the cells was usually well preserved. 48 hours after infection, especially in cells that had come off the glass spontaneously, nuclear membranes often had a wrinkled and fragmented appearance (Fig. 1). In a few instances, cellular fragments containing virus-like particles were seen (Fig. 9).

Lesions of the Cytoplasm.—Cells infected 24 to 26 hours earlier and having virus-like particles in their nuclei showed no lesion of the cytoplasm that could be distinguished from that of uninfected controls. After 48 hours, it was noticed that many but not all cells with intranuclear particles had unusually large numbers of irregular electron-dense granules in the cytoplasm (Fig. 1). Mitochondria and small vesicular forms of ergastoplasm (endoplasmic reticulum)[5] were also present at this time. In a few instances, dissolution of the cells was manifested by a scattered extracellular distribution of cytoplasmic organelles.

Uninfected Cells.—Control observations were carried out with cells treated in the same manner as infected ones except that virus was omitted. In addition, cells in stages of early active growth were studied because of the possibility that another viral agent might be associated in some manner with these carcinoma cells.[6] No virus-like particles were found in nuclei of uninfected cells, but the cytoplasm of rapidly growing cells contained many unusual globoid bodies (Fig. 2). These structures are illustrated in a separate paper (37), and reasons are given to indicate that they may be forms of mitochondria.

Large irregular electron-dense granules easily distinguished from the globoid bodies but identical in appearance with those found in the cytoplasm of infected

[5] We do not have an opinion as to which term should be adopted for general use.

[6] Virus-like particles have been demonstrated in tumor cells by electron microscopy. Examples and references are given in recent articles (34–36).

cells were also present singly or in small numbers in the cytoplasm of uninfected cells. Occasionally large numbers of such granules were present in localized portions of uninfected cells particularly when the cells were kept in maintenance solution at 30°C. To investigate the possibility that such dense granules might be manifestations of cytoplasmic degeneration caused by various deleterious conditions including viral infection, cells were allowed to stand in balanced salt solution without any serum or nutrient for 2 days at 30°C. At the end of this time, the cells had a rounded or granular appearance, and sections examined in the electron microscope showed marked distortions of nuclear and cytoplasmic structure. Nevertheless, no increase of electron-dense granules resulted.

Some other observations of uninfected cells may be summarized as follows:—

The nucleoplasm consisted of fine granular material evenly distributed and readily distinguished from the virus-like particles of infected cells (Fig. 2). One or more large nucleoli usually were present in each nucleus, and, especially in rapidly growing cells, often were vacuolated. Nucleoli were composed of fine granules tightly packed together (Fig. 6). Mitotic figures were seen occasionally and were present even in cells in maintenance solution at 30°C. Cells with mitotic figures were lacking in nuclear membranes; chromosomes appeared as masses of fine granules (Fig. 2).

Mitochondria were almost always much less dense than those previously observed in the bronchial epithelium of the mouse (23) and showed characteristic internal structure. In some cells or portions of the cytoplasm, only a few scattered mitochondria were seen; in other places, they were extremely numerous.

The ergastoplasm (endoplasmic reticulum) of HeLa cells consisted mainly of small round and ovoid forms; membranes were seen only rarely, and small particles were not prominent. No difference appeared to exist between the ergastoplasm of rapidly growing cells and those kept for 1 to 2 days in maintenance solution.

Numerous examples were observed of structures corresponding to the Golgi complex (38–40). These formations were distinctive in appearance and could be easily recognized among other structures in the cytoplasm. Sometimes they appeared in a somewhat circular form similar in size to the globoid structures (37).

Irregular microvilli were a prominent feature of the cell membranes.

Light Microscopy.—Observations of stained preparations with the light microscope were made in order to assist interpretation of the electron micrographs.

Application of the Feulgen method to uninfected cells grown on slides showed nuclei scattered fairly uniformly over the fields, usually singly (Fig. 14). The nuclear membranes were thin, and fine granules were scattered diffusely throughout the nucleoplasm except that regions occupied by nucleoli were Feulgen-negative and were surrounded by delicate Feulgen-positive membranes (Fig. 17). A few nuclei in slides of uninfected cells were smaller than most of the others and were more deeply stained. Many mitotic figures were seen even with uninfected cells kept in maintenance solution.

Nuclei in slides of infected cells were seen to occur in clumps (Figs. 15 and

16) and to be almost uniformly small and deeply stained. Nuclear structure of such cells resembled the few small hyperchromatic nuclei in slides of uninfected cells.

In groups of cells infected 18 to 26 hours previously, the Feulgen method revealed a small proportion of cells with intranuclear inclusion bodies. Nuclei containing these bodies were distinguished from others by a lack or paucity of fine granules within the nucleoplasm and by a slight irregular thickening of the nuclear membrane. The bodies were large and were often surrounded by clear areas (Figs. 18 to 21). Most of the intranuclear bodies failed to take the Feulgen stain, but significant numbers were found to be Feulgen-positive (Fig. 19).

Electron-dense, irregular granules in the cytoplasm were thought to consist mainly of lipide because of the affinity of osmium tetroxide for lipide-containing structures. However, staining for lipide with oil red 0 revealed numerous granules in the cytoplasm which became much larger when the cells were allowed to stand in balanced salt solution without serum or nutrient for 2 days, while the electron-dense granules remained unchanged.

Mitochondrial stain of uninfected cells showed that most of the mitochondria of each cell were concentrated in a single paranuclear area.

DISCUSSION

It seems almost certain that viral particles have been identified in the nuclei of infected cells in this investigation. In addition to the fact that the particles were not found in uninfected cells, their discrete uniform appearance and their frequent occurrence in crystal-like arrangement served as distinctive characteristics. Similar findings have been reported by Swedish workers (41) who have found particles in crystal-like formation within the nuclei of HeLa cells infected with a virus, probably of the same group, isolated from a mesenteric lymph node.[7,8]

In spite of the relatively small size of the intranuclear virus-like particles, it has been possible to show internal bodies and limiting membranes. Resemblance of this type of internal structure to that of large viruses (33, 49–51) gives further indication of the viral nature of the intranuclear particles.

Estimations of particle size by comparison with latex spheres of known size have indicated mean maximal diameters of 64 and 67 mμ. These figures are considerably less than ones obtained by methods of ultrafiltration, ultracentrifugation, and electron microscopy of purified preparations shadowed

[7] Morgan, Howe, Rose, and Moore have confirmed the presence of virus-like particles in nuclei of HeLa cells infected with adenoviruses and have shown remarkable illustrations of the crystalline formations (42, 43).

[8] Crystal-like formations have been seen in sections of cells infected with other viruses (44–46) and in purified preparations of viruses (27, 47, 48).

with uranium (52).[9] It seems likely that this difference in observation of particle size might be explained by the dissimilarity of the experimental methods.[10] Nevertheless, we are attracted to the possibility that the viral particles may be surrounded by an electron-lucent capsule that would be invisible in sections of cells but would affect measurements of purified particles covered with metal deposited by the procedure of shadow casting.

The intranuclear location of virus-like particles facilitated their recognition because the visualized structure of the nucleus was simpler than that of the cytoplasm. For this reason, it is possible that a few scattered viral particles in the cytoplasm may have escaped detection. Nevertheless, the presence of many particles in the nucleus appears to be significant and indicates that the biochemical environment of the nucleus is especially suitable for viral growth. It might be expected, also that mechanisms of viral release from the cell might be affected by intranuclear location and thereby account for the relatively low yields of virus from infected cultures (54) and also for the fact that cultivation of cells in tissue culture may be needed in order to detect viral activity (55).

The fact that only a few nuclei of cultures infected for 24 hours showed virus-like particles suggests that viral growth had taken place in only a minority of cells during this time interval. Although the failure to see virus-like particles in a nucleus might be explained by the possibility that the plane of the section did not include them, we think that the fraction of cells with visible particles was so small as to render this possibility unlikely. Furthermore, it seems reasonable to assume that nuclei with inclusion bodies and margination of chromatin were sites of viral growth, and such cells were also few in number. Therefore, since light microscopic evidence indicates that practically all of the cells participate in the cytopathogenic effect, it seems probable that cytopathogenicity due to these viruses is primarily a toxic phenomenon (56) and is related to the agglutination of cells by viruses (57). In this connection, it may be noted that the cytopathogenic effect of influenza virus on HeLa cells is not accompanied by reproduction of the infectious form of the virus (58), and also that cytopathogenicity and viral multiplication can be dissociated in the case of HeLa cells and poliomyelitis virus (59).

SUMMARY

HeLa cells were infected with adenoviruses (types 1–4) and sectioned for electron microscopy after intervals of 20 to 48 hours. Clusters of virus-like

[9] Values obtained by these methods were as follows: Ultracentrifugation 109 ± 6 mμ. Ultrafiltration 80 to 120 mμ. Electron microscopy of purified virus shadowed with uranium 90 ± 4 mμ.

[10] For example, a slight increase in size of latex spheres may occur after exposure to the beam of electrons (53).

particles were found within the nuclei of infected cultures but not in those of uninfected controls. The particles were often arranged in rows as if in crystalline formation. Maximal diameter of particles was approximately 65 mμ, and internal bodies were demonstrated.

Lesions of infected cells included target-like structures of the nuclear membrane, large nuclear vacuoles (type 2), and increased numbers of large irregular electron-dense granules in the cytoplasm 48 hours after infection. Examination of infected cultures by light microscopy, using the Feulgen reaction, showed intranuclear inclusion bodies and a cytopathogenic effect consisting of clumping of cells without pyknosis of nuclei. A lipide stain showed numerous cytoplasmic granules that were not identical with the large, irregular, electron-dense granules of the cytoplasm.

Practically all the cells showed the viral cytopathogenic effect, but only a minority of cells were found to contain virus-like particles or intranuclear inclusion bodies.

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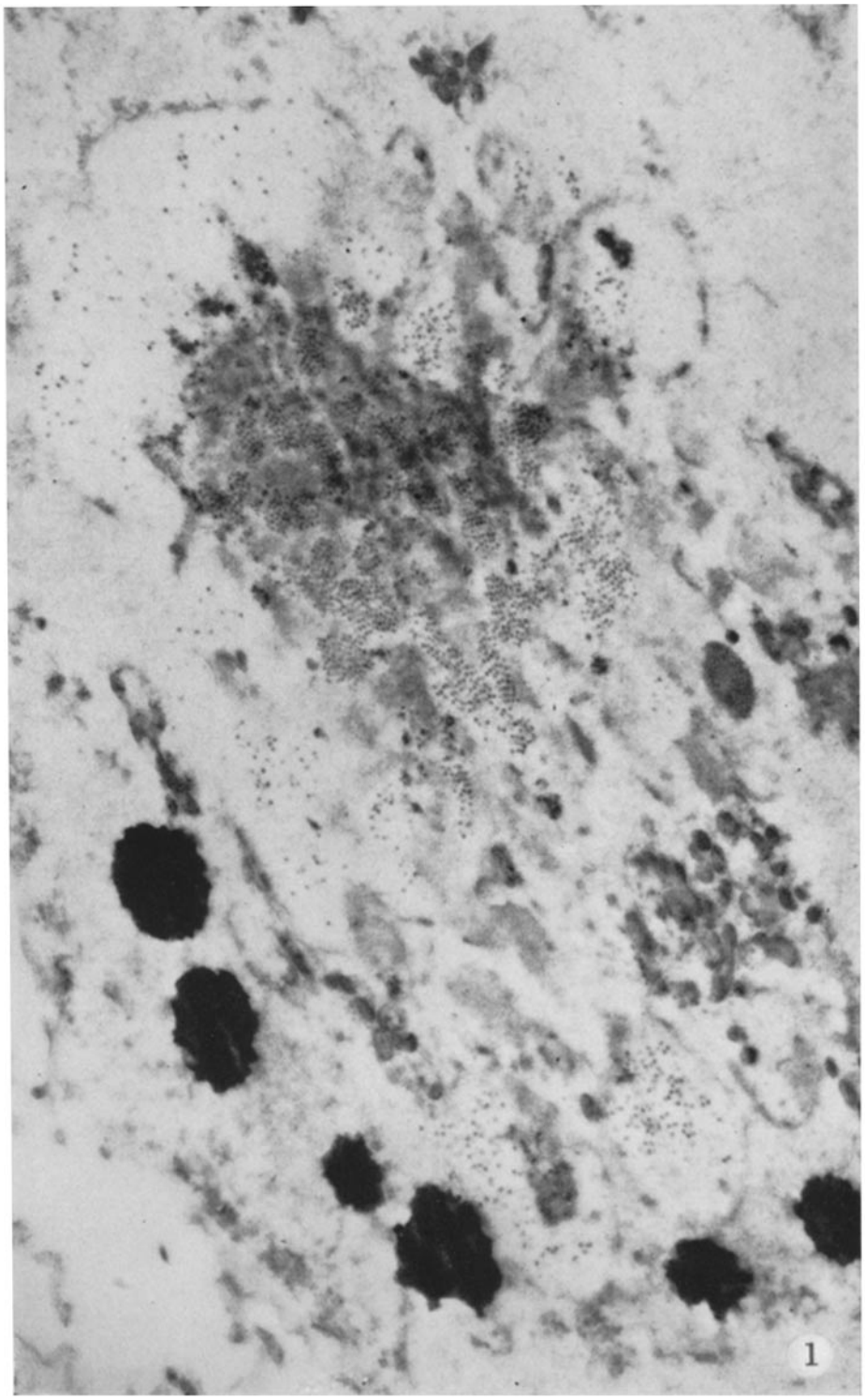
EXPLANATION OF PLATES

Cells shown were fixed in situ with Palade's fluid for 30 minutes except as noted.

PLATE 36

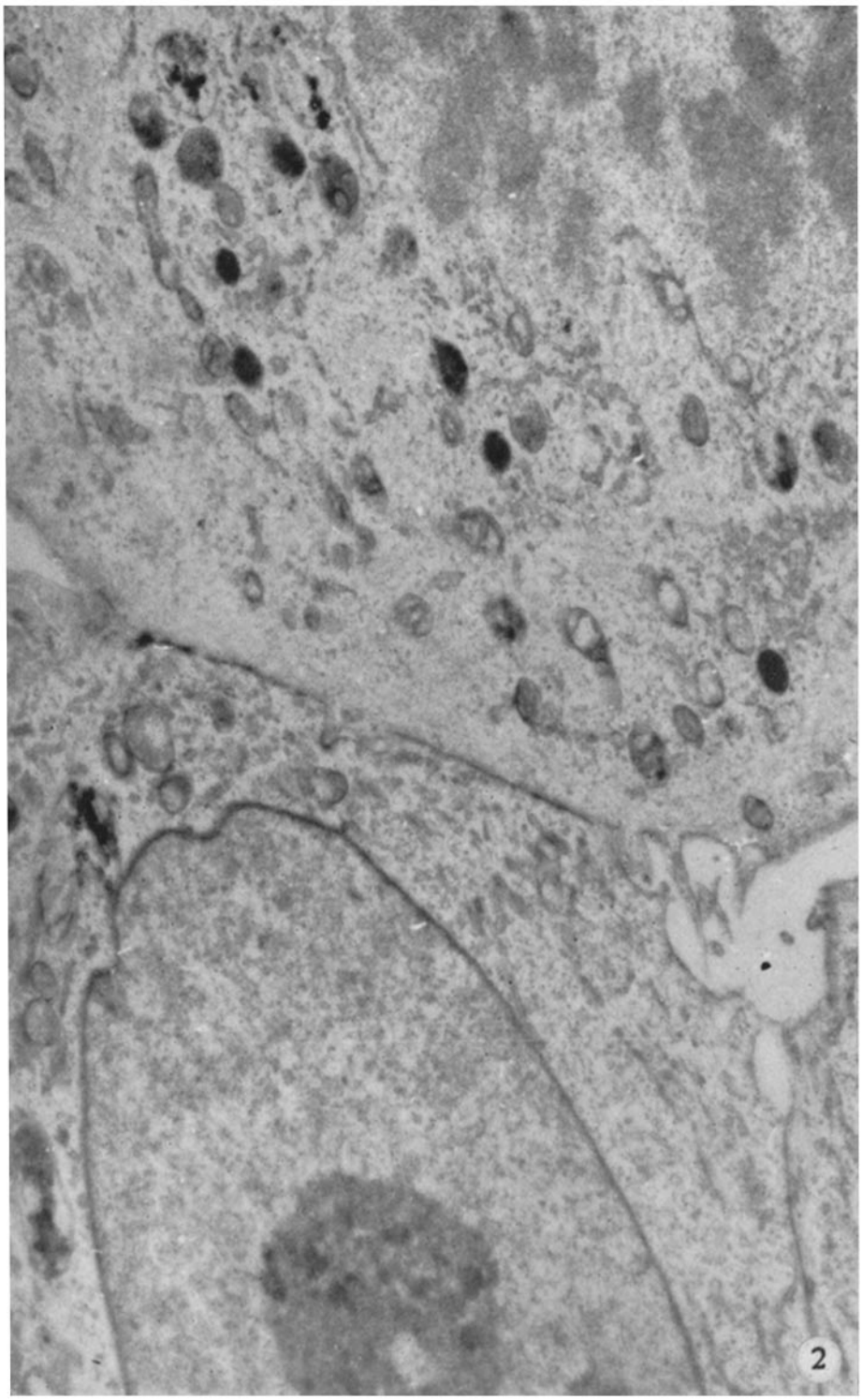
FIG. 1. Clusters of virus-like particles within the nucleus of a HeLa cell 47 hours after infection with type 4 virus. In the center of the nucleus, virus-like particles are frequently arranged in rows and are in close apposition with moderately electron dense nuclear material. At the periphery of the nucleus, virus-like particles are somewhat scattered. The nuclear membrane is discontinuous and not visible in some places. Cytoplasmic organelles are scanty. Large irregular electron-dense granules are present in the cytoplasm. Cells were removed from the glass with sodium citrate and treated with Palade's fixative for 1 hour. Magnification approximately 14,250.



(Harford *et al.*: Adenoviruses by electron microscopy)

PLATE 37

FIG. 2. Uninfected HeLa cells. In the lower part of the micrograph, the nucleus and nucleolus of a cell are seen. The cell in the upper part of the micrograph is in mitosis and shows sections of chromosomes. Cytoplasms of both cells contain mitochondria, globoid bodies, and ergastoplasm. Magnification approximately 22,500.

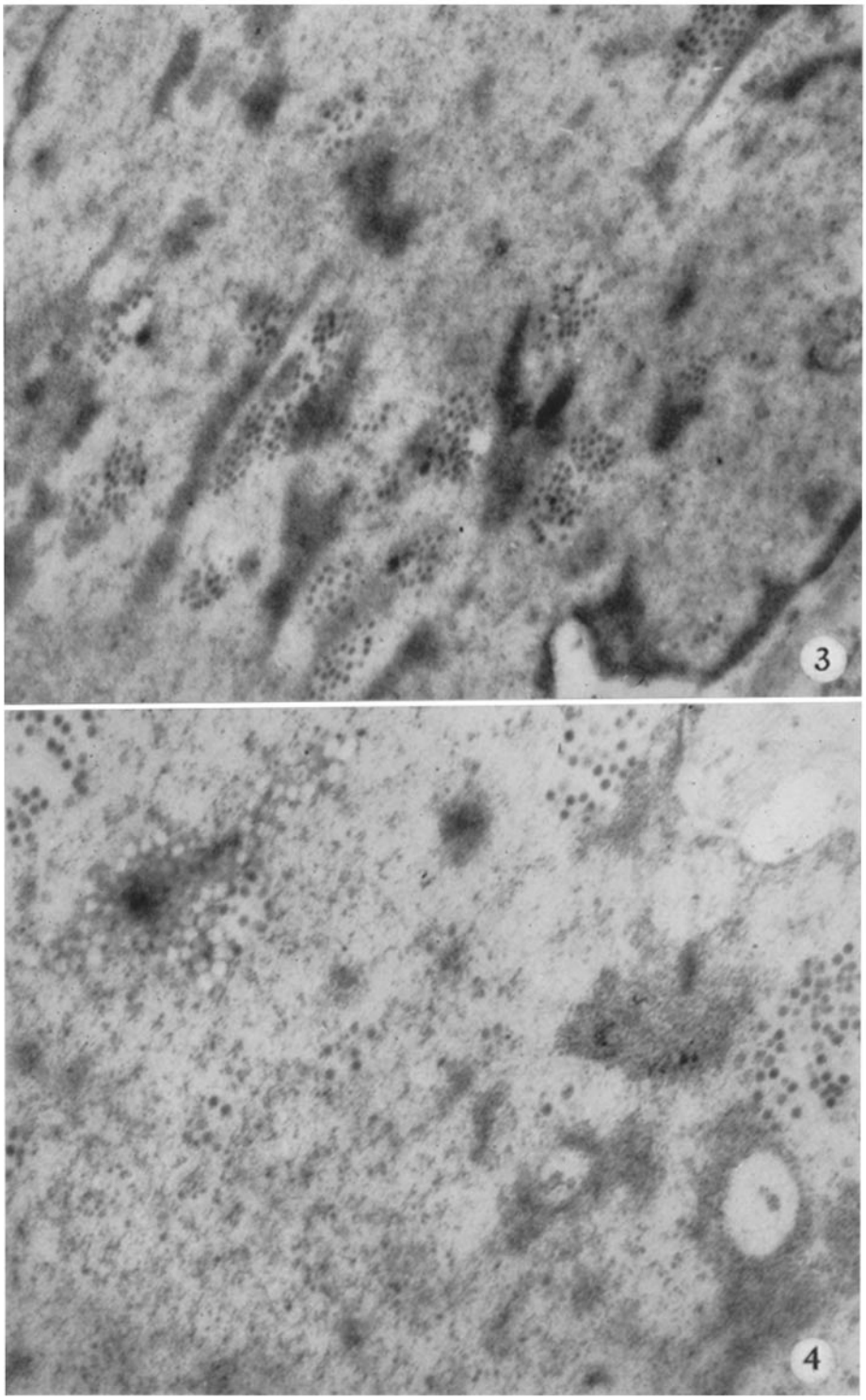


(Harford *et al.*: Adenoviruses by electron microscopy)

PLATE 38

FIG. 3. Clusters of virus-like particles in the nucleus 24 hours after infection with type 3 virus. Clusters are associated closely with moderately electron-dense nuclear material. The nuclear membrane is in the lower right corner. Magnification approximately 22,500.

FIG. 4. Clusters of virus-like particles in the nucleus 24 hours after infection with type 3 virus. The nuclear membrane is in the upper right corner. A target-like structure is present in the nuclear membrane in the lower right corner. Formation of nuclear material in a pattern resembling a honey comb is present in the upper left portion of the micrograph. No similar pattern was seen in many other preparations. Magnification approximately 32,500.

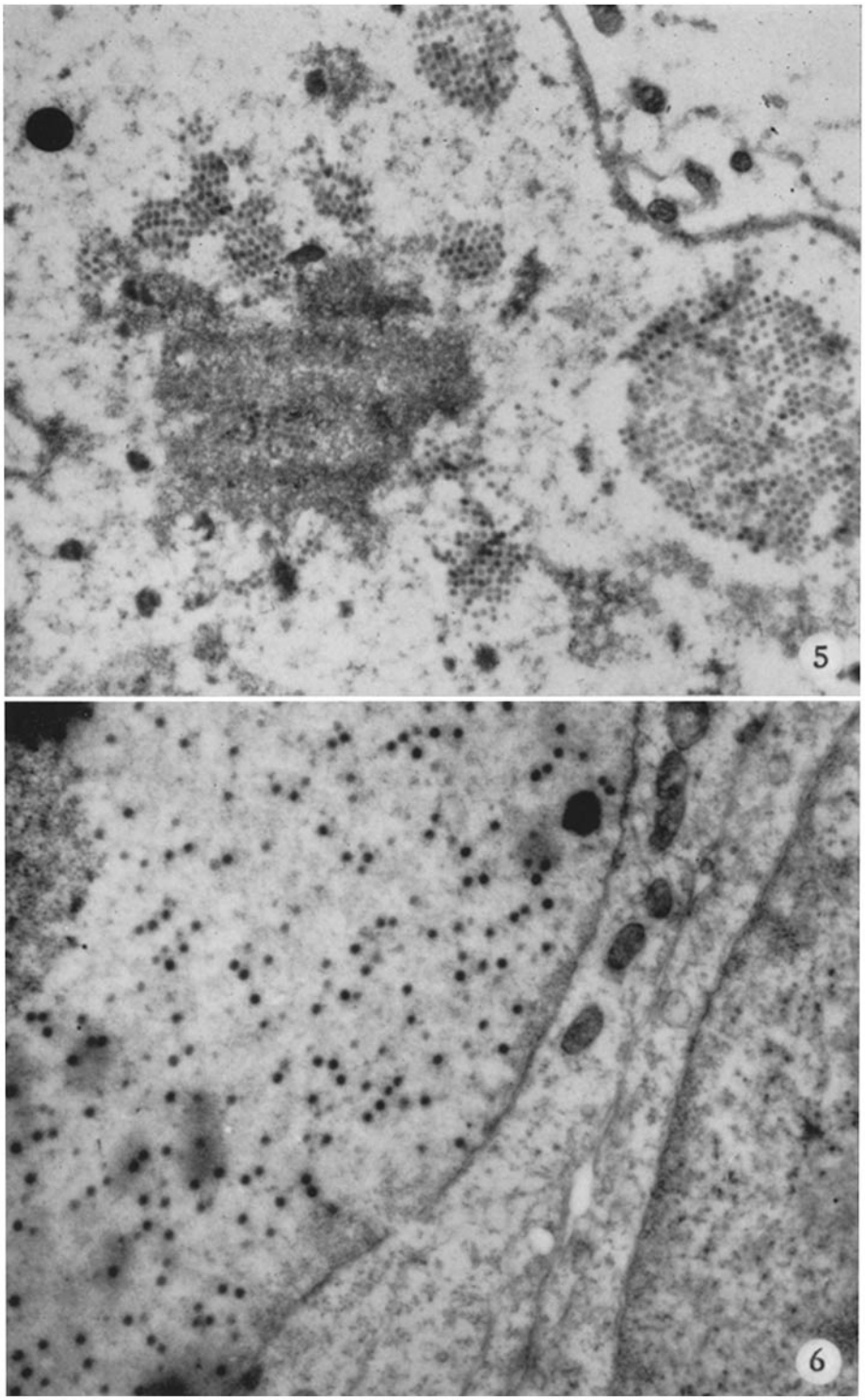


(Harford *et al.*: Adenoviruses by electron microscopy)

PLATE 39

FIG. 5. Clusters of intranuclear virus-like particles in crystal-like arrangement 26 hours after infection with type 4 virus. In the upper left corner is a latex sphere 340 mμ in diameter. The nuclear membrane is in the upper right corner. In the center of the micrograph is a mass of finely granular material resembling a nucleolus and containing a cluster of virus-like particles. Spectroscopic V-O plate. Magnification approximately 22,500.

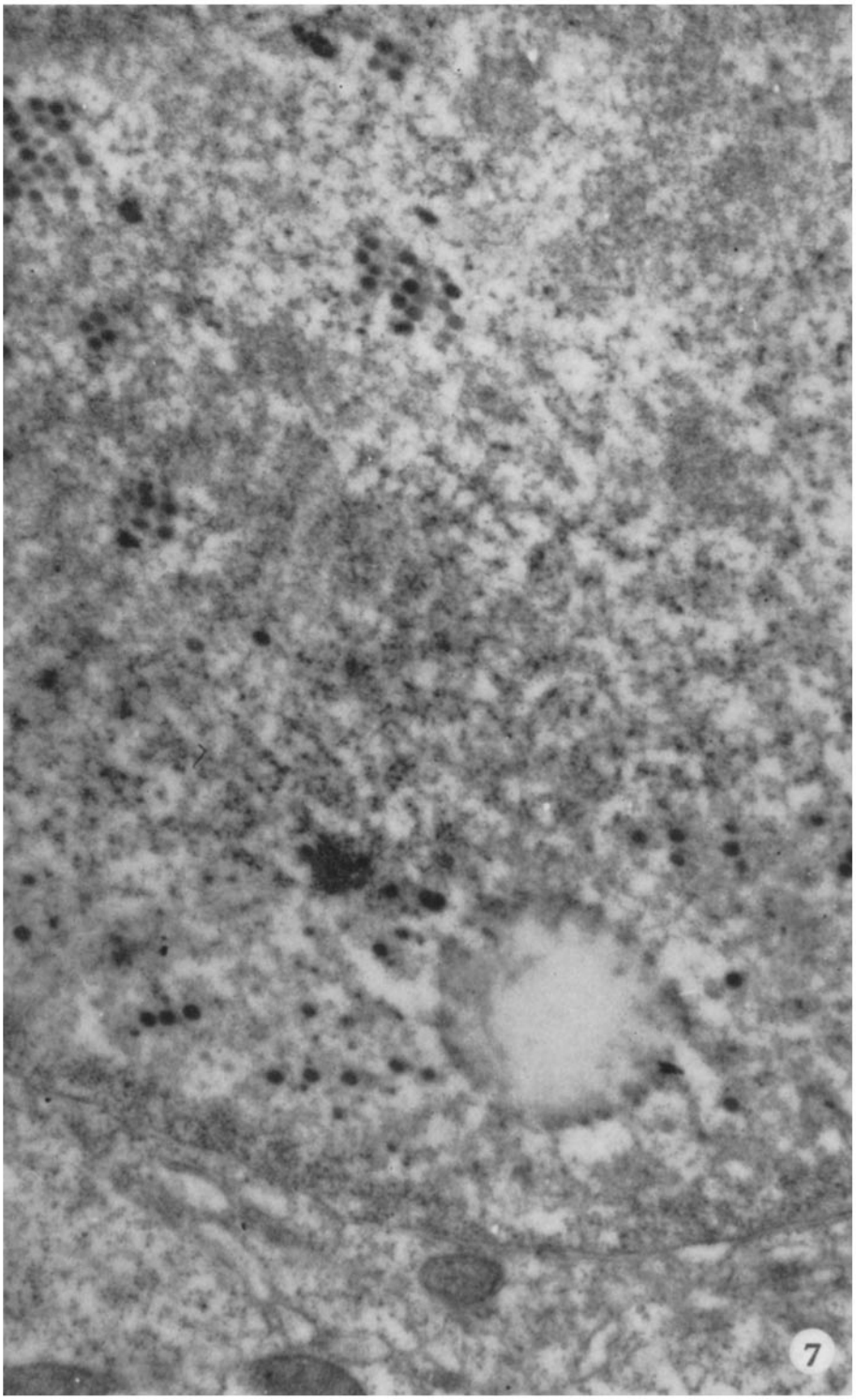
FIG. 6. The left portion of the micrograph shows widely spaced virus-like particles within a nucleus 24 hours after infection with type 2 virus. Around most of the virus-like particles, the nucleoplasm is moderately electron lucent. The nucleus on the right side of the micrograph does not show virus-like particles. Adjacent cell membranes and cytoplasms of the cells extend from the upper right corner to the lower center of the micrograph. The fine granules in the upper left corner appear to be those of a nucleolus. Magnification approximately 28,500.



(Harford *et al.*: Adenoviruses by electron microscopy)

PLATE 40

FIG. 7. Clusters of intranuclear virus-like particles in the upper part of the micrograph 24 hours after infection with type 2 virus. In the lower part of the micrograph is a large intranuclear vacuole with indistinct margins. The nucleoplasm adjacent to the vacuole contains some scattered virus-like particles. The nuclear membrane and adjoining cytoplasm are below the vacuole. Magnification approximately 34,500.



(Harford *et al.*: Adenoviruses by electron microscopy)

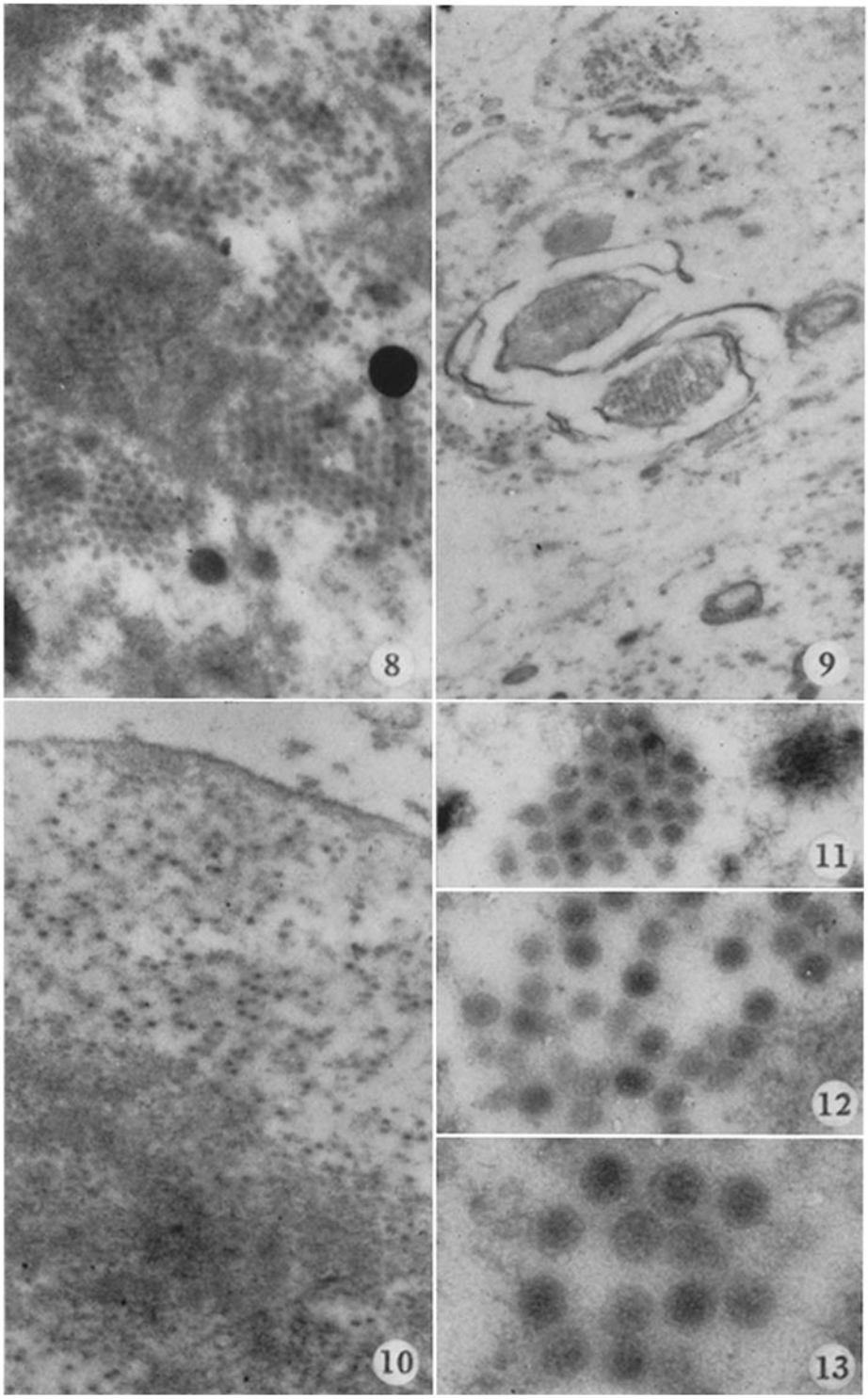
PLATE 41

FIG. 8. Intranuclear virus-like particles in crystal-like arrangement 26 hours after infection with type 4 virus. Virus-like particles are also present within the moderately electron-dense material near the center of the micrograph. A latex sphere 340 mμ in diameter is seen on the right. Spectroscopic V-O plate. Magnification approximately 28,500.

FIG. 9. Two clusters of virus-like particles in crystal-like arrangement and partially surrounded by discontinuous membranes. The general architecture of the cell was altered to such an extent that areas of nucleus and cytoplasm could not be distinguished. 48 hours after infection with type 3 virus. Cells were removed from the glass with sodium citrate and treated with Palade's fixative for 1 hour. Magnification approximately 14,250.

FIG. 10. Virus-like particles within the nucleus 48 hours after infection with type 1 virus. The nuclear membrane is in the upper portion of the micrograph. Magnification approximately 22,500.

FIGS. 11, 12, and 13. Virus-like particles with enveloping membranes and internal bodies. Twenty-six hours after infection with type 4 virus. Fig. 11. Magnification approximately 45,600. Fig. 12. Magnification approximately 104,000. Figure 13. Magnification approximately 130,000.



(Harford *et al.*: Adenoviruses by electron microscopy)

PLATE 42

Cells were grown directly on glass slides, stained by the Feulgen method, and counterstained with orange G.

FIG. 14. Uninfected HeLa cells distributed fairly uniformly over the field. Some mitotic figures and a few small hyperchromatic nuclei are present. × 110.

FIG. 15. A high percentage of cells are clumped and have small hyperchromatic nuclei. 25 hours after infection with type 3 virus. × 110.

FIG. 16. Nearly all the cells are clumped and have small hyperchromatic nuclei. 24 hours after infection with type 2 virus. × 110.

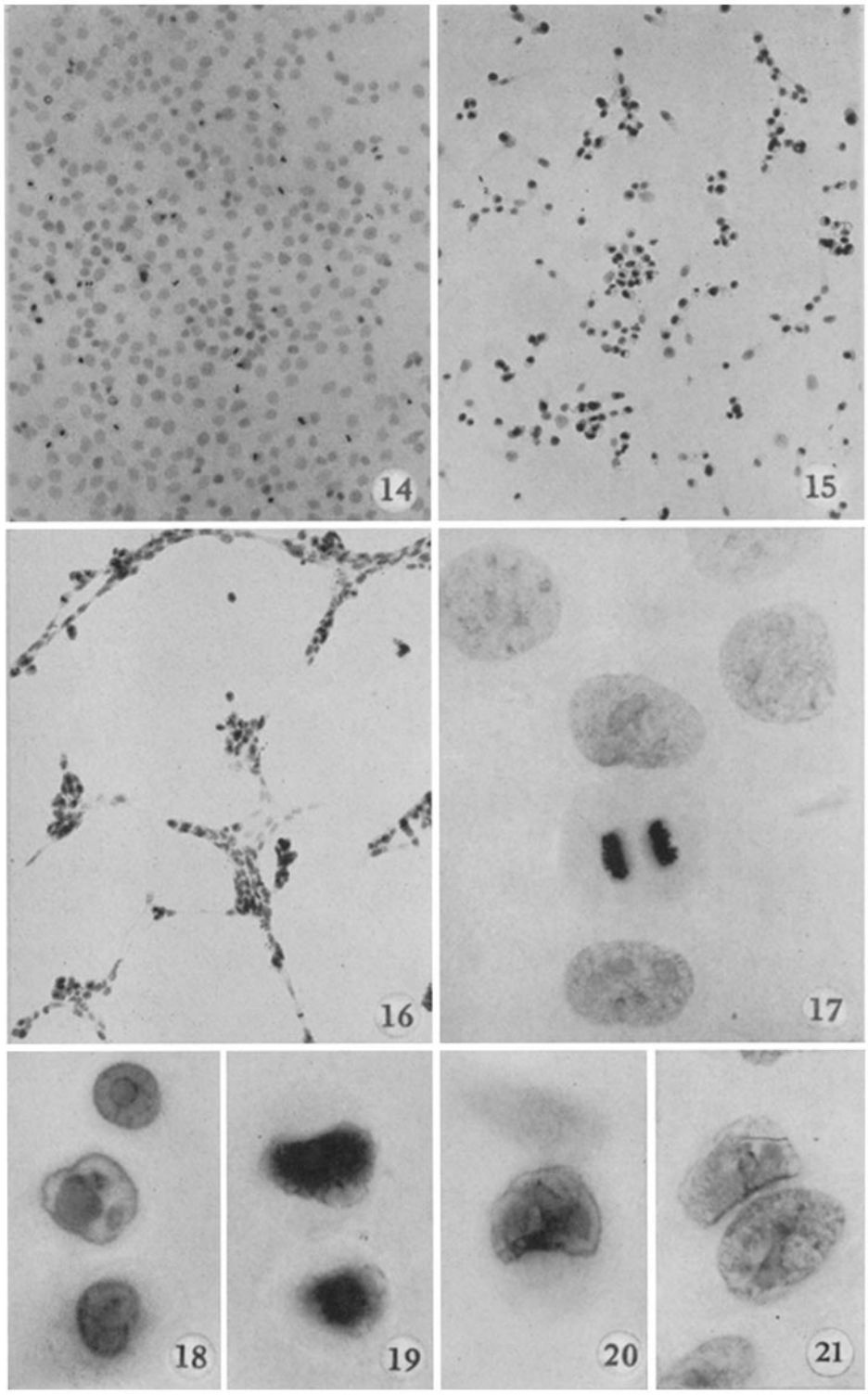
FIG. 17. Nuclei of uninfected HeLa cells. × 1350.

FIG. 18. The central nucleus shows margination of chromatin, a large Feulgen-negative intranuclear inclusion body, and two nucleoli. The other two nuclei are small and hyperchromatic. 25 hours after infection with type 3 virus. × 1350.

FIG. 19. Two nuclei containing large Feulgen-positive masses 20 hours after infection with type 4 virus. × 1350.

FIG. 20. Nucleus with marginated chromatin and a large Feulgen-negative intranuclear inclusion body. 18 hours after infection with type 1 virus. × 1350.

FIG. 21. Of the two nuclei in the center of the field, the upper one shows margination of chromatin and a large Feulgen-negative inclusion body. The lower nucleus is only slightly hyperchromatic. 24 hours after infection with type 2 virus. × 1350.



(Harford *et al.*: Adenoviruses by electron microscopy)