A CORRELATED HISTOCHEMICAL AND ELECTRON MICROSCOPIC STUDY OF THE INTRANUCLEAR CRYSTALLINE AGGREGATES OF ADENOVIRUS (RI-APC VIRUS) IN HELA CELLS*· ‡

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Plates 1 to 6

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INTRODUCTION

The adenoviruses, formerly designated RI-APC viruses[1], a group comprising at least 18 serologically distinct types, have been shown to be the causative agents of several human disease entities, including acute undifferentiated respiratory disease, epidemic keratoconjunctivitis, and pharyngoconjunctival fever. These agents readily infect and propagate in HeLa cell cultures, and certain types form intranuclear crystalline aggregates as revealed by the electron microscope (1–3). These crystal-like formations are composed of a close packed lattice of regularly ordered spherical viral particles each of which measures about 60 mμ. They have been described in detail by Morgan *et al.* (3), whose electron microscope studies have suggested the probable stages in the intracellular growth and aggregation of the virus. These investigators found that viral particles develop first in the nucleus. With the increased formation of virus, aggregation or crystallization takes place; viral particles are subsequently released and ultimately find their way into the cytoplasm.

Some of the same structures seen in electron micrographs can be characterized chemically *in situ* by employing consecutive sections for electron microscopy and for histochemical tests. In the present study, this procedure was followed in an attempt to relate histochemical findings with the morphological changes during infection of the HeLa cell with viral types 3, 4, or 7.

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[1] The term adenoviruses has been proposed by a committee on nomenclature (*Science*, **124**, 119, 1956) and has been generally accepted.

Materials and Methods

HeLa cells were cultured in stock bottles in a nutrient medium of either 75 per cent "tris" solution (lactalbumin hydrolysate, dextrose, yeast extract with tris (hydroxymethyl) aminomethane buffer in Earle's balanced salt solution, adjusted to pH 7.7.), 15 per cent human serum and 10 per cent horse serum, or of 85 per cent "tris" solution, 10 per cent human serum and 5 per cent horse serum. Cells were transferred to tubes after dislodgement with 0.01 per cent trypsin and washing with Earle's saline. Each tube received approximately 30,000 cells suspended in 0.4 ml. of a maintenance medium consisting of 85 per cent "tris" solution and 15 per cent horse serum. After incubation for 2 to 3 days at 37°C., each tube was inoculated with 0.1 ml. of diluted RI-APC virus suspension, types 3, 4, or 7, and reincubated from 18 to 96 hours. Cells were collected, washed in balanced salt solution, and centrifuged to form small pellets. The pellets were fixed in 1 per cent osmium tetroxide, buffered at pH 7.2, and brought to 0.34 osmolar concentration with sodium chloride. They were then dehydrated in increasing concentrations of ethyl alcohol and embedded in partially polymerized butyl methacrylate, polymerization of which was completed at 80°C. Sections were cut with glass knives on a Porter-Blum thermal expansion microtome. Sections used for electron microscopy were floated on an acetone-water mixture, mounted on formvar-coated copper grids, and examined in an RCA type EMU 2E electron microscope.

For comparative examination of structures with the electron and light microscopes, alternate thin (about 0.05 μ) and thick (about 2 to 3 μ) sections were cut. The latter were affixed to glass slides, the methacrylate, removed with chloroform, and the preparations then rehydrated, bleached in 3 per cent hydrogen peroxide in 70 per cent alcohol, and stained (q.v.). Maps were then made by photomicrographing all fields and reconstructing the entire section on a montage. A record of all the cells present in the section and their relationships was thus obtained with the phase microscope. Maps were similarly constructed from electron micrographs. Cells that could be located on these maps by both phase and electron microscopy were chosen for study. DNA was stained with the Feulgen technique, using a hydrolysis time of 18 minutes. Suitable control tests were performed on other similar sections. Some specimens were then counterstained with 0.025 per cent azure B bromide at pH 4.

OBSERVATIONS

The stages of infection to be described are those reconstructed by Morgan *et al.* (3). Presumably the earliest morphologic stage of intranuclear infection is recognized by the presence of dense osmiophilic granular material within the nucleus, which at first contains no recognizable viral particles. Within or contiguous to this material, the individual viral particles develop. Reticulated condensations of the dense granular "matrix" are shown in Fig. 1. This material (*a*) is not stained with the Feulgen technique applied to the adjacent section (inset, Fig. 1) and is thereby distinguishable from chromatin (*b*). The density in the photomicrograph represents osmium deposition and contours resulting from difference in refractive index of material and mounting medium, but there is no fuchsin color in the dense material (*a*) in the center of the nucleus. The chromatin in the peripheral part of the nucleus is faintly to moderately stained.

When the viral particles become sufficiently concentrated in any locus they form small aggregates in which they are arranged in crystalline array. These microcrystals are at first spatially related to the dwindling dense matrix ma-

terial. In Fig. 4 they are identifiable in the electron micrograph (arrows), and, in the adjacent thick section for light microscopy, appear as small intensely Feulgen-positive granules (arrow) in association with a matrix which itself is not colored by the Feulgen test.

The small crystals may grow, or coalesce to form large crystals or ordered aggregates. The crystals are composed of 60 mμ virus particles in the orientation of a cubic body centered lattice (Low and Pinnock, cited in reference 3). In their growth by accretion, the viral aggregates displace the finely divided nuclear chromatin material. They may sometimes grow to occupy more than half the area of the nucleus.

These crystals are intensely stained by the Feulgen test for DNA. There is good correspondence between the crystalline masses of the electron micrograph and the large Feulgen-stained masses of the adjacent thick (2 to 4 μ) section used as a histochemical preparation (Figs. 2 to 5). Fig. 3 is an electron micrograph at about 9,000 diameters of a typical well formed rhomboidal crystal with flat faces and angular corners in the nucleus. The particles of the cut face are regularly disposed. The adjacent 2 μ section clearly shows the same crystal intensely stained by the Feulgen procedure. Comparison of the 0.05 μ and the 2 μ sections reveals the extent of the compression that may be sustained in cutting sections of considerable thinness for electron microscopy.

In nuclei in which the development of aggregates is far advanced, there is a displacement of nuclear chromatin toward the inner aspect of the nuclear membrane, and a disappearance of much of the chromatin from the neighborhood of aggregated virus. The chromatin, visible with the electron microscope as a finely divided rather dense granular material, is moderately (sometimes faintly) colored after the Feulgen reaction in the adjacent thick sections, as compared to the dense, intensely colored viral aggregates and crystals.

Crystalline aggregates may grow or disintegrate. Disintegration releases individual viral particles which tend to scatter toward the nuclear periphery, conferring a slightly less intense Feulgen color on this area than that given by chromatin alone. Probably at any stage, irregular crystals or scattered particles can erupt into the cytoplasm through breaks in the nuclear membrane. Such viral masses in the cytoplasm, like those in the nucleus are revealed as Feulgen-positive bodies the staining intensity of which seems to depend upon the density or degree of packing of the virus accumulations.

Sometimes the aggregations may disperse completely, while the nuclear membrane remains intact. The nucleus at this advanced stage becomes a sac of scattered viral particles and contains recognizable chromatin only at its margins (Fig. 5). Such nuclei in 2 to 3 μ sections are colored diffusely with the Feulgen stain (right inset, Fig. 5). The cytoplasmic organelles are relatively well maintained until advanced stages of intranuclear infection, but some evidence of cell injury is the rule once virus is released into the cytoplasm.

If the Feulgen-stained preparations are counterstained with azure B, the color of the latter dye preponderates over the rather light Feulgen color of the nuclear chromatin which thus appears dark blue. The viral aggregates, on the other hand, which are more intensely Feulgen-stained, bind little azure and retain a lavender-pink color. This permits a clear distinction between nuclear chromatin, which appears blue, and viral material, which is lavender-pink. The left inset of Fig. 5 and Figs. 6 to 8 illustrate such double stained preparations.

The left inset of Fig. 5 shows two nuclei whose chromatin is being displaced peripherally. Chromatin material is seen intermingled with the aggregates of virus, but is in no definite continuity with them. Rather, there tends to be a narrow, clear zone adjacent to some of the viral masses. In later stages (Fig. 8) the chromatin is entirely disposed against the inner aspect of the nuclear membrane as a narrow, sometimes incomplete ring, and would seem to be much reduced in amount. Larger or smaller aggregations of virus (Figs. 6, 7, and 8), which may take up the greater area of the nucleus, are readily distinguishable.

DISCUSSION

Some authors have proposed the use of thicker sections fixed, dehydrated, and embedded as for electron microscopy, for phase contrast microscopy (4) and direct light microscopy after suitable staining (5, 6). It has been shown that such sections can be suitably stained in a number of ways. In particular, the successful application of the Feulgen reaction to material fixed in buffered osmium tetroxide solutions (a stain previously known to be applicable after Fleming's solution (7)), has lately again been demonstrated (6, 8–10). The refinement of cutting thin sections for electron microscopy alternating with thicker contiguous sections for histochemical tests has already been successfully employed by Moses (10) in his study of chromatin. Some of the photomicrographs, especially Figs. 3 and 5, illustrate graphically the extent of deformation that may occur as thinner sections are cut. In the accompanying illustrations it is apparent that the thin sections may be compressed in one dimension up to about 40 per cent. In assessing the size of fixed objects, the thicker 2 to 3 μ section may also be assumed to have suffered distortion due to the knife. It is of interest in considering the changes in size and shape suffered by cells during preparation for electron microscopy, that recent observations (11) on some effects of fixation and dehydration of living neurons in culture have confirmed that while osmium tetroxide fixation causes no appreciable distortion or displacement, even careful and gradual alcohol dehydration can cause marked shrinkage and some small displacement of intracellular structures. Interpretation of electron micrographs should be made with these facts in mind.

The correlating technique described has permitted us to ascertain the presence of DNA in both the crystals and non-crystalline aggregates of adenovirus.

Because of the close packing of the particles in the crystals and the apparent absence of electron-dense material in the interstices between the spheres, it seems most unlikely that significant amounts of material other than that comprising the particles can gain hospitality. Indeed, crystallization may be regarded as a tentative criterion of viral purity (12). It may therefore be justifiable to regard histochemically demonstrable substances, *e.g.* DNA, in the crystalline aggregates ("inclusion bodies") as constituents of the viral bodies themselves.

The specificity and precision of localization of the Feulgen reaction for DNA are generally accepted (13, 14). The differences in stainability of nuclear and viral DNA in the Feulgen-azure preparations are thought to result from the difference in protein with which they are respectively associated, and will be discussed in some detail in a forthcoming report.

In consisting wholly (or almost wholly) of virus arranged in a definite pattern, the intracellular crystals of types 3, 4, and 7 of the adenovirus group are reminiscent of those of plant mosaic virus (15, 16). Crystal-like formations have been observed by differential centrifugation of material from human infectious skin papilloma (17, 18) as well as in sections of cells of this lesion (1, 19). The intranuclear inclusion bodies of some such cells have been found to contain DNA (20, 21) which is acquired during a developmental stage of the inclusion (22, 23). However, while the identification of the regularly arranged viral particles seen in the electron microscope with these intranuclear inclusion bodies is probable, it has not yet been made with certainty.

The presence of large amounts of DNA in the types of adenovirus investigated has been established in the present study for all stages in which recognizable particles appear. Further histochemical work, which will be reported subsequently, has revealed the presence of an associated protein of a non-histone type; no carbohydrate nor lipide could be detected. Once formed, the aggregates of adenovirus retain their tinctorial and histochemical characteristics until the complete dispersal of the aggregates into individual particles. There is no residual matrix material, and the mature inclusion bodies of adenoviral infection do not contain, at least in recognizable amount, the products of host cell reaction.

Wolman (24), in a survey of the Feulgen stainability of the inclusion bodies in many human and animal viral diseases, has concluded that almost all of these are positive during some stage of their evolution. Reported discrepancies in the Feulgen reaction in many of these inclusion bodies are doubtless attributable to the fact that the intracellular bodies undergo a series of progressive changes entailing different tinctorial characteristics with the development, multiplication, and dispersal of virus.

Studies correlating even more closely the successive temporal stages in the early intranuclear development of RI-APC virus with histochemical properties

of the intranuclear bodies would offer much desirable information. The evolution of DNA-containing viral particles in association with and seemingly at the expense of a dense matrix which does not itself contain DNA is a process which deserves further study. This process might prove to be similar to the acquisition of DNA by the apparently nucleic acid-free material of the early intranuclear inclusion body of human skin papilloma. It is also reminiscent of the production of virus from the "chromatic mass" in the nucleus of insect cells infected with polyhedral viral disease. The "chromatic body" which is said to be formed *de novo*, at first is Feulgen-negative; viral rods presumably containing DNA are formed in or from it (25, 26).

SUMMARY

HeLa cells in tissue cultures infected with types 3, 4, or 7 of adenovirus (RI-APC virus) were studied in order to correlate certain histochemical and electron microscopic findings.

Adjacent thin (*ca.* 0.05 μ) and thick (2–4 μ) sections of osmium-fixed, methacrylate-embedded cells were cut; by mapping the sections the same cells could be identified with both the electron and the light microscope.

Intranuclear crystalline aggregates seen with the electron microscope to be composed of ordered arrays of viral particles were found by means of the Feulgen reaction to contain DNA. DNA is therefore assumed to be a constituent of the viral particle. The virus appeared to develop from an osmiophilic Feulgen-negative matrix. Displacement of nuclear chromatin occurred during this process.

A Feulgen-azure staining method was found to permit clear distinction between viral and nuclear (host) DNA in thick sections.

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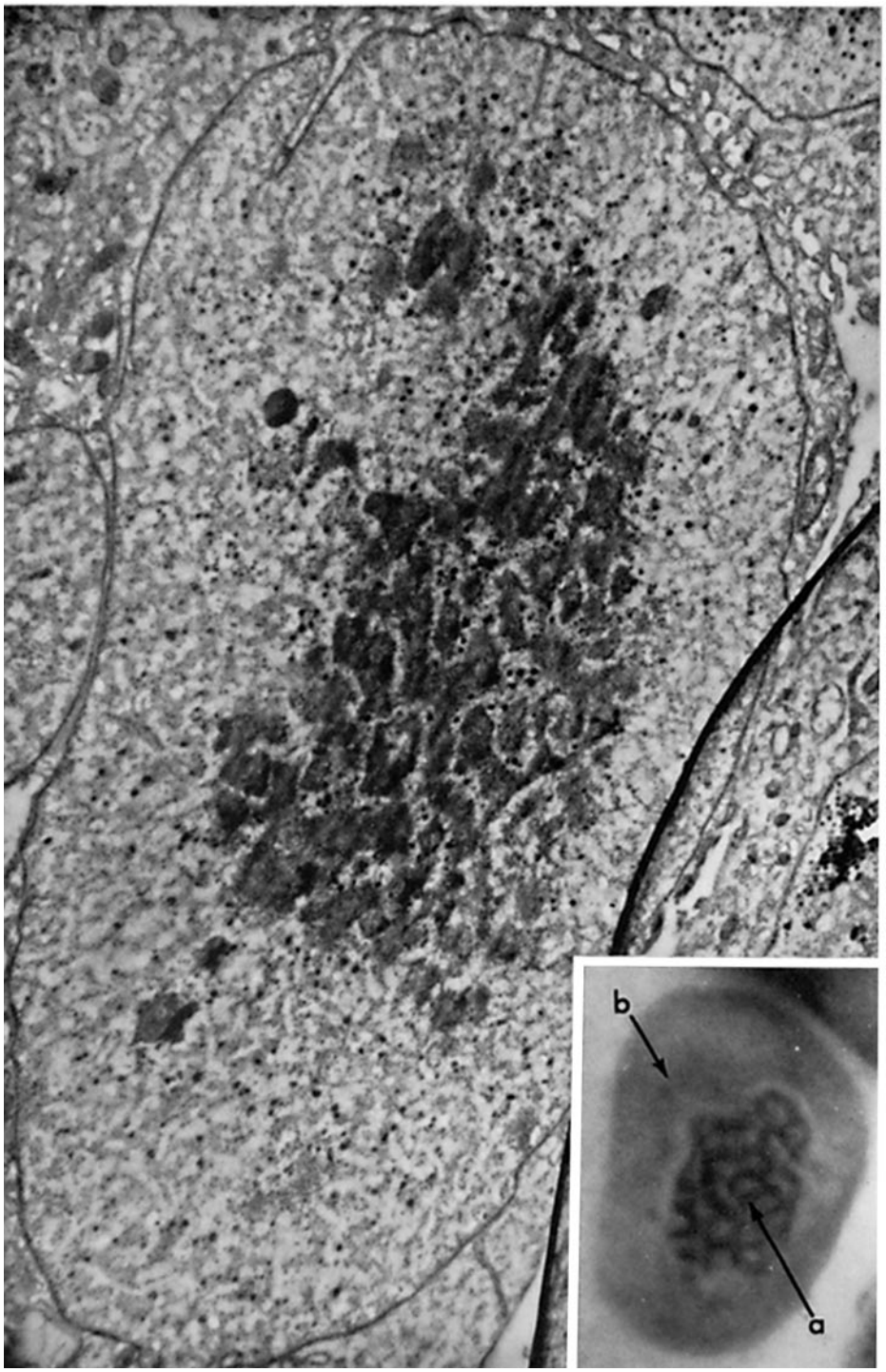
EXPLANATION OF PLATES

PLATE 1

FIG. 1. Electron micrograph of the nucleus of a HeLa cell in a presumably early stage of adenovirus (RI-APC virus) infection. Reticulations of dense osmiophilic material occupy the center of the nucleus. Nucleoli have disappeared. Virus apparently forms in connection with and at the expense of this dense material. The dark line traversing the lower right side of the field is an artefact. × 13,000.

Inset. The same nucleus as it appears in the adjacent thick (2 to 4 μ) section after Feulgen staining. The reticulated cords of dense material (*a*) are not colored by the Feulgen stain but appear dark in the photomicrograph because of the deposited osmium and the difference in refractility between this material and the mounting medium. The peripheral part of the nucleus, (*b*), appearing grey in the photomicrograph, is moderately colored with the Feulgen reaction. × 4,000.



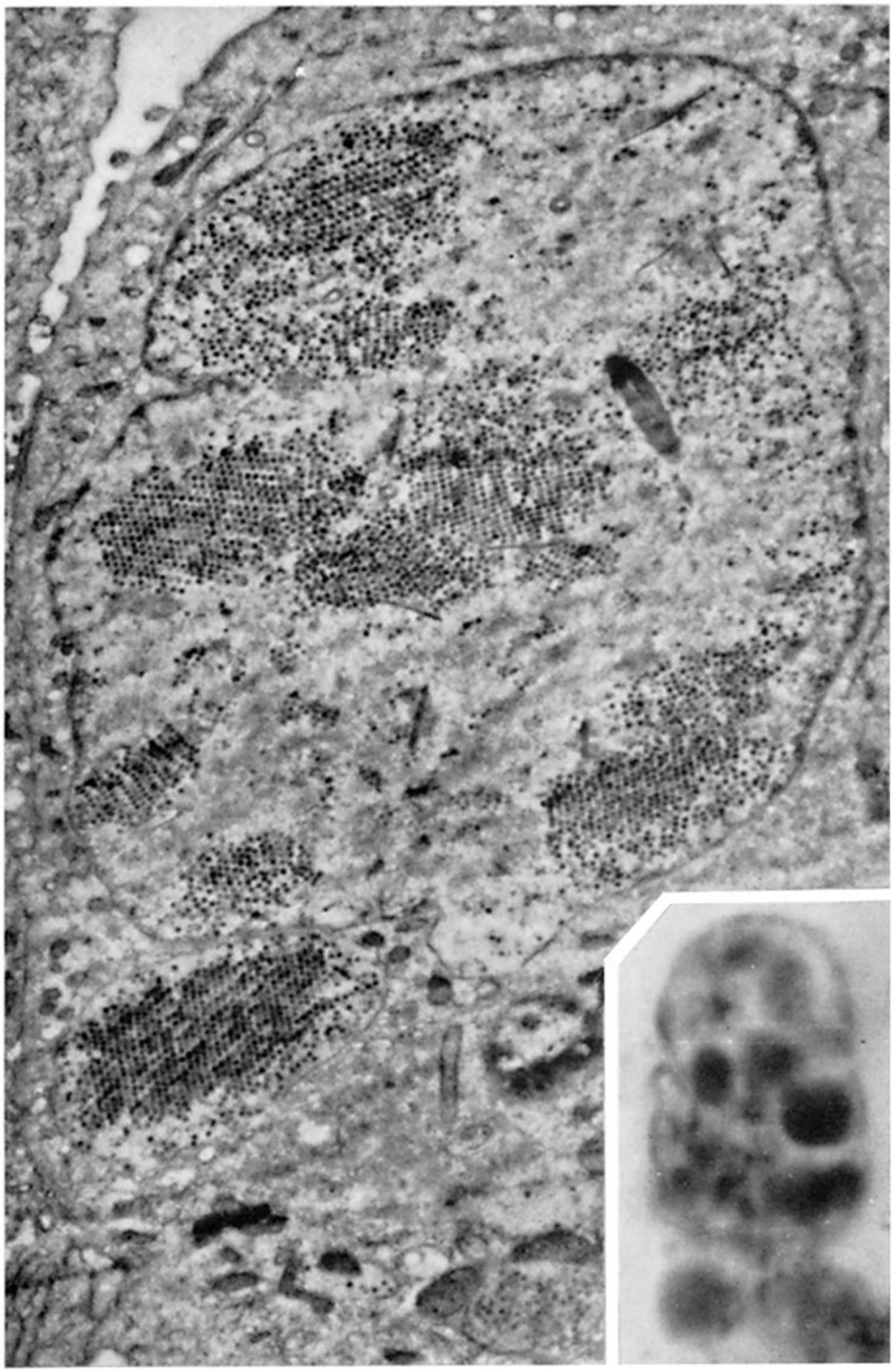
(Bloch *et al.*: Intranuclear adenovirus in HeLa cells)

FIG. 1

PLATE 2

FIG. 2. Electron micrograph of a cell at a later stage of infection showing intranuclear aggregates and crystals composed of viral particles. Each of the particles measures about 60 mμ in diameter. × 14,000.

Inset. Adjacent thick section of the same nucleus, showing some of the aggregates seen in Fig. 3. These are intensely stained by the Feulgen test. Additional aggregates, which lie outside the plane of the thin section, appear in the thick section. × 4,500.



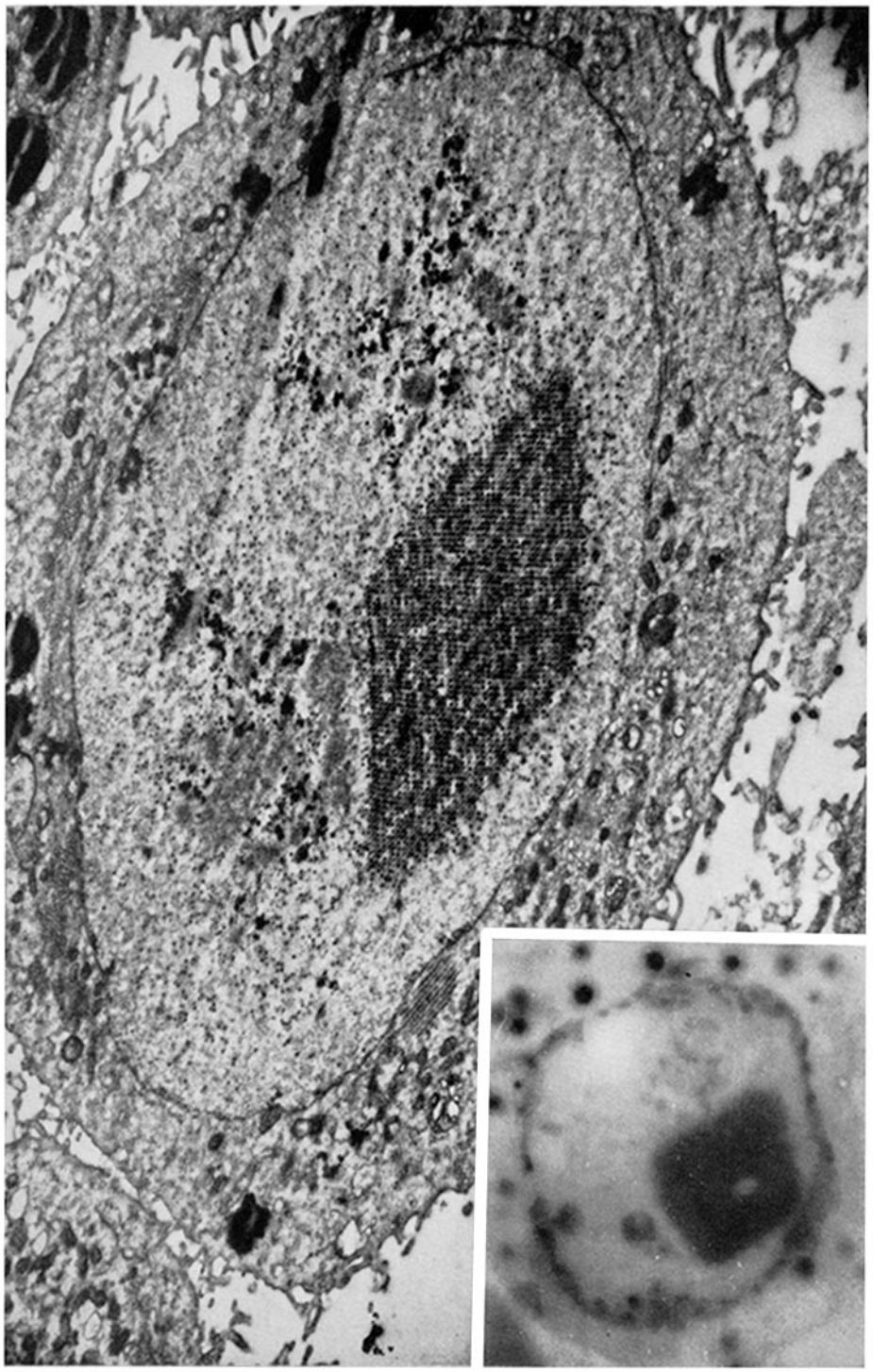
(Bloch, *et al.*: Intranuclear adenovirus in HeLa cells)

FIG. 2

PLATE 3

FIG. 3. Electron micrograph showing a well formed crystal of rhomboidal profile in the nucleus of an infected cell. Adjacent to it are two masses of osmiophilic material in association with some scattered viral particles. × 9,000.

Inset. Contiguous thick section showing the same crystal, which is strongly colored by the Feulgen reaction. The masses of dense osmiophilic material are visible in the same relation to the crystal. × 3,700.



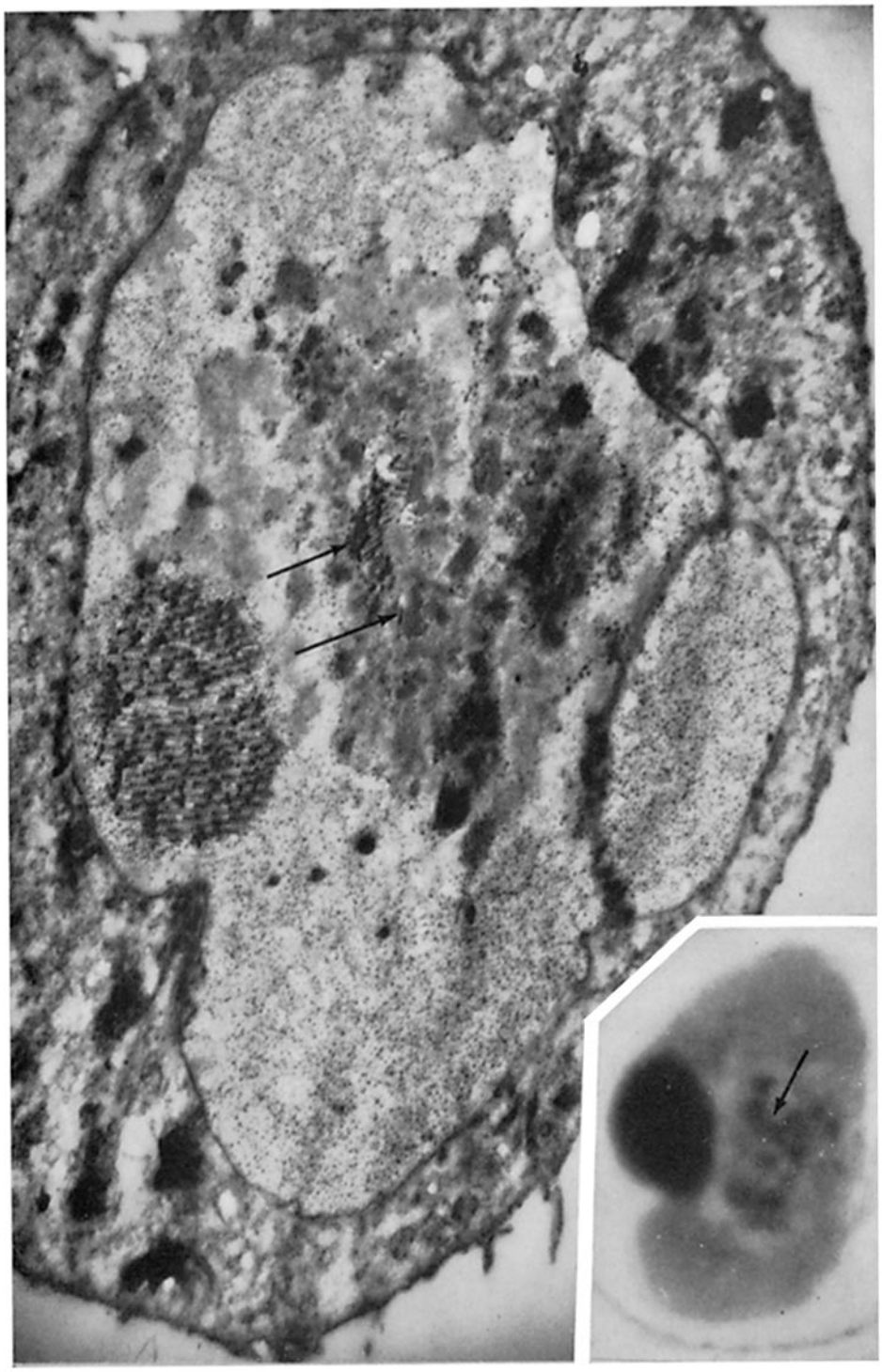
(Bloch, *et al.*: Intranuclear adenovirus in HeLa cells)

FIG. 3

PLATE 4

FIG. 4. Electron micrograph of a cell showing simultaneously several stages in the development of intranuclear virus. Scattered viral particles and microcrystals, two of which are indicated by arrows, lie among masses of dense osmiophilic material. A large crystalline aggregate, apparently undergoing dissociation into discrete viral particles, is seen to the left of the osmiophilic material. Individual viral particles are scattered throughout the remainder of the nucleus. × 9,000.

Inset. Adjacent thick section showing microcrystals as Feulgen-stained granules (shown by arrow) lying within an osmiophilic matrix, which therefore appears relatively lighter in the micrograph. The large crystal is seen as an intensely Feulgen-stained body. The regions containing dispersed virus appear homogeneous and are moderately stained by the Feulgen test. × 2,700.



(Bloch, *et al.*: Intranuclear adenovirus in HeLa cells)

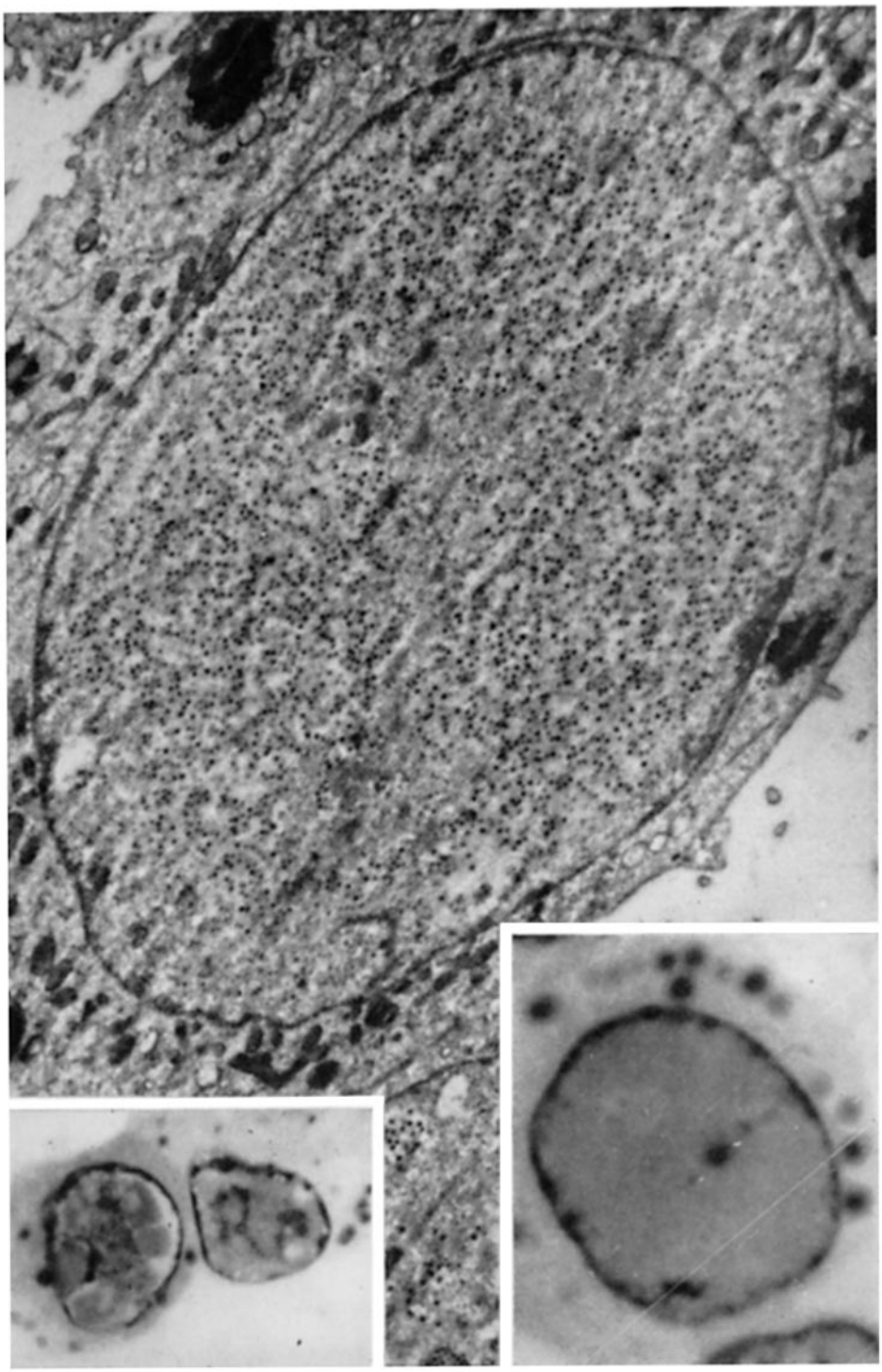
FIG. 4

PLATE 5

FIG. 5. Electron micrograph showing virus in the dispersed phase within an intact nuclear membrane. × 12,000.

Right Inset. Adjacent thick section showing a fairly homogeneous nuclear interior which has been moderately stained by the Feulgen reaction. Intensely osmiophilic lipide globules in the cytoplasm assist in proper orientation of the sections. The orientation is not immediately apparent because of the deformation which has occurred in cutting the thin section. × 4,100.

Left Inset. Photomicrograph of infected cells stained with the Feulgen-azure method for DNA. The DNA of the viral aggregates which stains lavender-pink, is shown in the photomicrograph as light grey; the DNA of the nuclear (host) chromatin stains blue and is shown in black. × 3,000.



(Bloch, *et al.*: Intranuclear adenovirus in HeLa cells)

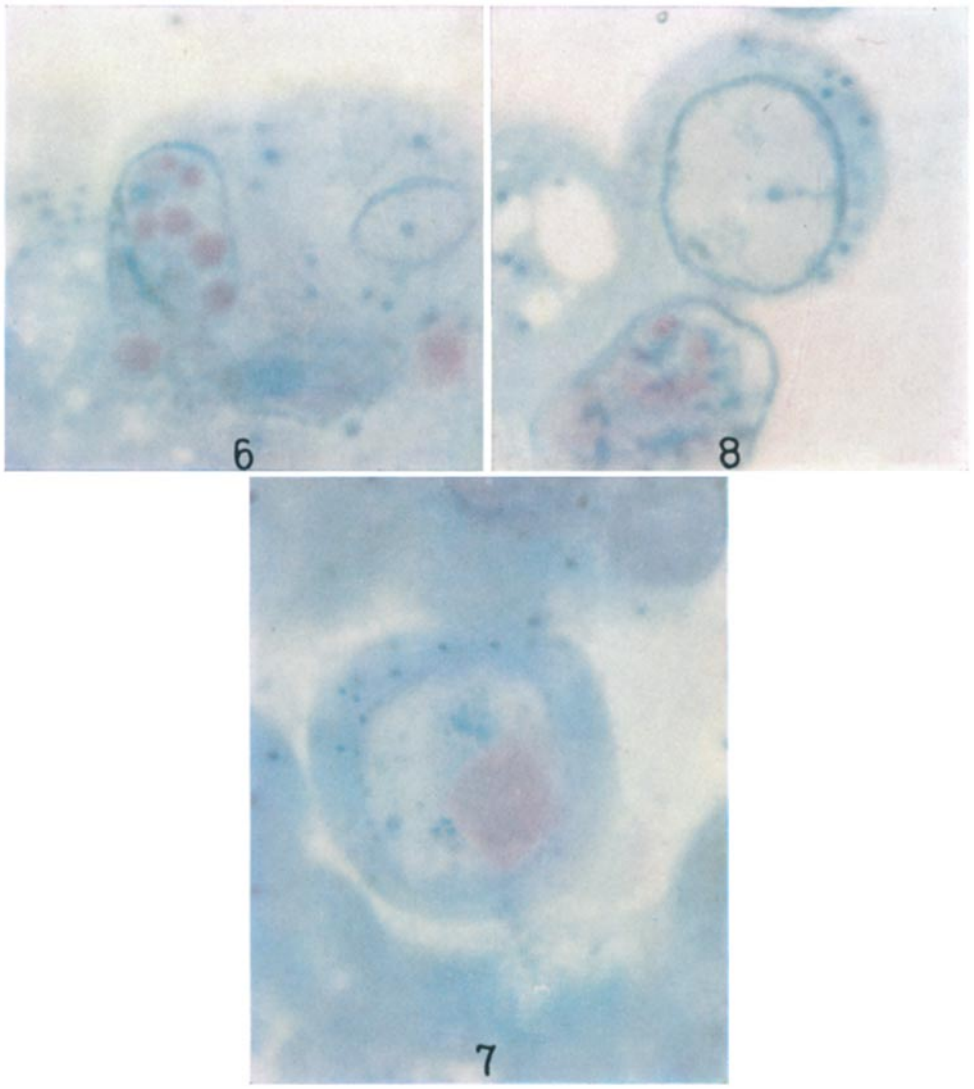
FIG. 5

PLATE 6

FIG. 6. Same cell as that shown in Fig. 2, stained with the Feulgen-azure double staining procedure. Nuclear chromatin stains blue, viral aggregates lavender-pink. Cytoplasmic material appears green. This and the ensuing color photographs are of cells sectioned at 2 to 3 microns; the color intensity is therefore somewhat diminished. × 1010.

FIG. 7. Same cell as that shown in Fig. 3, stained with the Feulgen-azure double staining procedure. × 1250.

FIG. 8. Same cell as that shown in Fig. 5, stained with the Feulgen-azure double staining procedure. A neighboring cell is shown containing the lavender-pink viral aggregates and the blue nuclear chromatin. × 1200.



(Bloch, *et al.*: Intranuclear adenovirus in HeLa cells)