AN ELECTRON MICROSCOPE STUDY OF THE DEVELOPMENT OF SV40 VIRUS

NICOLE GRANBOULAN, M.D., P. TOURNIER, M.D., R. WICKER, and W. BERNHARD, M.D.

From the Institut de Recherches Scientifiques sur le Cancer, Villejuif (Seine), France

ABSTRACT

Kidney cells, predominantly from *Cercopithecus* monkeys but also from baboons, were infected *in vitro* with the SV40 virus. The infectious cycle was studied with the electron microscope by means of thin sections of cells fixed from 3 hours up to 11 days after infection. The frequency of virus formation and various nuclear and cytoplasmic lesions in relation to the infection are described. The virus particles appear in the nucleus in close contact with the chromatin. In a small number of cells they have been observed as early as 10 to 12 hours after infection, but most often they appear 24 to 48 hours afterward. Their mean diameter is 33 mμ. They have no membrane and are frequently arranged as crystal-like structures. In addition to the appearance of virus, one observes various lesions in the nucleoplasm and particularly in the nucleolus, which shows an early hypertrophy and produces unusual, dense condensations in contact with the nucleolonema. The importance of these nucleolar lesions and the relationship between the SV40 virus and the polyoma, common wart, and Shope papilloma viruses are discussed.

The SV40 virus was discovered by Sweet and Hilleman (38) in *Macacus rhesus* kidney cells, being identified by the cytopathic effect it produces on the kidney cells of *Cercopithecus aethiops sabaeus*, a monkey which is not naturally a carrier of this viral agent. The cytopathic effect is characterized by an intense cytoplasmic vacuolization after several days of infection ("vacuolating agent").

Our preliminary electron microscope study on *Cercopithecus* kidney cells *in vitro* infected by SV40 virus revealed that the site of development of the virus is in fact entirely nuclear, that the cytoplasmic vacuolization is actually the consequence of its multiplication in the nucleus, and, finally, that its morphological structure is similar to that of the polyoma virus (40). We also observed the importance of nuclear and nucleolar lesions (alterations produced by virus infection) which appear in the course of the infection. These first results are in agreement with the observations made by Gaylord and Hsiung (15). When it was discovered that the inoculation of this agent into newborn hamsters produced tumors of the sarcoma type (13, 17), this virus acquired importance in tumor virus research. A more detailed morphological study of the multiplication of this virus and the cellular lesions it induces appeared necessary to provide a better understanding of the cell-virus interaction following infection with an oncogenic agent.

In the course of our study we have paid particular attention to nucleolar alterations during the first stages of infection. In addition, we have been much interested in the comparison of the evolution of this virus with that of the polyoma agent.

MATERIAL AND METHODS

Cell Cultures

Primary cultures were prepared from kidneys of the African green monkey (*Cercopithecus aethiops sa-*

baeus) and the baboon (*Papio papio*). After trypsinization, the cells were grown in prescription bottles, in a medium containing 0.5 per cent lactalbumin hydrolyzate and 0.1 per cent yeast extract in Hanks's balanced salt solution, supplemented with 10 per cent calf serum. When the monolayers were completed, they were changed to a maintenance medium containing the same quantities of lactalbumin hydrolyzate and yeast extract in Earle's solution, supplemented with 1 per cent calf serum.

Virus Inoculation and Titration

The strain of SV40 virus was obtained from Dr. M. R. Hilleman. The virus stocks were grown in cells derived from the kidneys of baboon. Virus titration was performed by a plaque procedure derived from the Dulbecco technique for polyoma virus (12). Monolayers of green monkey kidney cells (300,000 cells per ml of medium, 5 ml per dish) were grown in 6 cm diameter plastic Petri dishes (Falcon). After one rinse with PBS, different dilutions of virus were put on the monolayers and left 40 minutes for absorption (42). The overlay (10 ml) was made with the same maintenance medium described above, but containing 1 per cent calf serum and 0.8 per cent agar. The dishes were incubated at 37°C in a box containing a mixture of air with 5 per cent CO_2. On the 11th day the cells were stained for 3 hours with neutral red (0.05 gm per liter lactalbumin medium), 5 ml per dish. The final count of the plaques was carried out on the 14th day.

The titer of the different stocks of virus varied between 5×10^6 and 10^8 plaque-forming units (pfu) per ml. The inoculum per prescription bottle containing 3 to 5×10^6 cells was either high (10^8 pfu) so as to assure an infection multiplicity of greater than 1, and hence simultaneous infection of a majority of the cells, or low (10^4 pfu) to obtain more spread out stages of the infection in one culture. This study deals with cellular changes induced with the low titer of virus concentration.

Infected tissue culture cells were prepared for electron microscopy from 3 hours to 11 days after infection. Non-infected cells from the same batch served as controls and were examined at similar intervals. After centrifugation at 2000 RPM for 10 minutes, the cells were fixed with 2 per cent osmium tetroxide buffered according to Millonig (36) and embedded in Epon. The ultrathin sections, prepared with a Porter-Blum microtome, were stained either 1 hour with a 5 per cent aqueous solution of uranyl acetate or by double staining (25, 31): 15 minutes in uranyl acetate followed by 15 minutes in lead acetate according to Karnovsky, method A (27). Furthermore, in order to carry out cytochemical studies on thin sections, several samples of *Cercopithecus* kidney cells, 24 hours and 48 hours after the infection, and non-infected cells of a control culture were also fixed with acrolein 5 per cent (29) for 20 minutes and embedded in water-soluble glycolmethacrylate (29). The ultrathin sections were stained with 5 per cent uranyl acetate at pH 5 for 45 minutes. The grids were examined with a Siemens Elmiskop I (80 kv, objective aperture 50 μ).

RESULTS

Cercopithecus Kidney Cells

Non-infected Cells

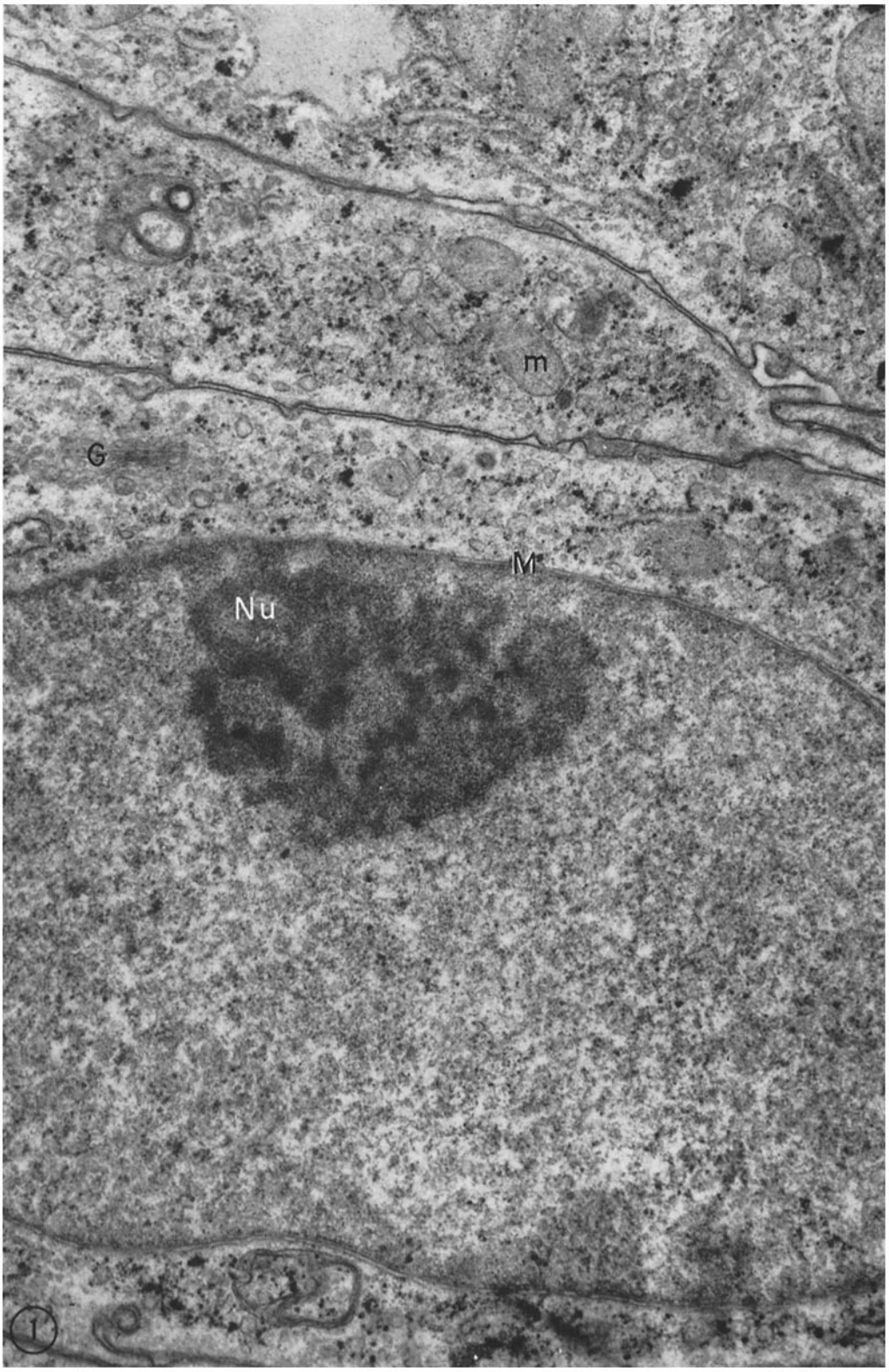
In culture the monkey kidney cells are in general irregularly rounded. Large nuclei account for the high nucleocytoplasmic ratio that they exhibit. The nuclear chromatin is rather homogeneous and the nucleoli, one or sometimes two, often appear in close contact with the nuclear membrane. The nucleolonema with some portions of increased contrast is easily visible, and in its loose meshes appears the finely granular "pars amorpha" (Fig. 1). The cytoplasm of these cells contains many free RNP granules, ergastoplasmic lamellae, a chiefly lamellar Golgi apparatus, and numerous mitochondria. Besides this cellular type, which is the most frequent one, there also exist elongated cells of the fibroblast type. We have not found significant ultrastructural changes, particularly with respect to the nucleolus, during 11 days of culture.

Development of SV40

From the 3rd to the 6th hour after infection most of the cells have the same appearance as the non-infected cells. However, in a few nuclei there is a slight hypertrophy of the nucleolus. This may result both from an increase in the amount of nucleolar material and from a loosening of the nucleolonema.

FIGURE 1

Non-infected *Cercopithecus* kidney cell. Homogeneous distribution of the nuclear chromatin. Nucleolus (*Nu*) with filamentous nucleolonema and pars amorpha close to the nuclear membrane (*M*). *G*, Golgi apparatus; *m*, mitochondrion. The dense cytoplasmic granules represent glycogen stained with lead. × 30,000.


m
G
M
Nu
1

At the 10th hour, the alterations are chiefly nuclear and nucleolar, but they involve only a small number of cells. First there is a distinct hypertrophy of the nucleus and, in particular, of the nucleolus. Two nucleoli are encountered more often than in the non-infected cells. The nucleolar lesions affect primarily the nucleolonema and are of two types: (*a*) The network of the nucleolonema is distinctly looser than in the controls (Fig. 3). In extreme cases the nucleolus appears as if it had been torn asunder. (*b*) Some parts of the nucleolonema retain their normal density, whereas other parts, of varying size and randomly distributed, exhibit an increased density (Fig. 3). It seems probable that the latter originate from the dense part of the nucleolonema in normal controls. This fine structure reveals tiny fibrillar elements. Preliminary studies with enzymic digestion suggest that they are composed of proteins and RNA.

In some hypertrophied nuclei, one or several extremely dense, regularly rounded or ovoid clusters of very fine granules are found (Fig. 3). They are often localized in proximity to the nucleolus.

At the 12th hour, besides the lesions already described, some nucleoli show another type of alteration characterized by fragmentation of the nucleolonema. The areas of increased density become separated from one another and isolated in the nuclear mass (Fig. 2, *e*). It has to be mentioned that, in very rare instances, even by the 10th and 12th hour of infection, a few nuclei contain a considerable number of viral particles filling all the nuclear area with formation of crystal-like arrangements, as will be described below.

After 24 hours, the cells with hypertrophied nuclei and nucleoli are still more numerous. The dense areas of the nucleolonema are now very frequent, polymorphous, and of much greater density (Figs. 4 and 5). They often form horseshoe- or sausage-shaped figures (Figs. 4 and 5). These are located within the nucleolus and at its periphery, from which they may become detached. No serial sections have been made. Much more rarely, the nucleolar condensations are small, spherical, and separated from the nucleolar material by a clear halo (Fig. 2, *d*). One also observes nuclear condensations similar to those described in the stage of the 10th or 12th hour, but they appear less dense and are often localized at the periphery of the nucleolus. They do not exist in control cells.

At 36 hours after the infection, the nuclei and the nucleoli have the same appearance as after 24 hours, but viral particles are more frequent. At this stage the cytoplasm may already be modified by the appearance of small vacuoles.

At 2 days after the infection one frequently finds some nuclei containing a large number of viral particles (Fig. 6). With the occurrence of viruses in the nuclei, the homogeneous distribution of the chromatin disappears, the nuclear mass being filled with viral particles. Only a few small fragments of nuclear chromatin persist, and these are generally closely surrounded by viruses. In such nuclei nucleolar hypertrophy has largely disappeared. The nucleolus has become smaller and contains in some areas an extremely dense substance formed by the accumulation of a finely granular material (Fig. 6). However, in a great number of nuclei which do not yet contain any detectable virus particles, or only a few, the nucleolar hypertrophy still persists with numerous, intense condensations.

In the nucleoplasm of some of these nuclei there exist one or several well delimited, spherical,

FIGURE 2

Different aspects of nucleoli during the development of SV40 virus in cultures of *Cercopithecus* kidney cells.

a. Nucleolus of non-infected cells.

b. Nucleolus after 12 hours of infection. Slight hypertrophy and enlargement of the intranucleolar meshes. Increase of size of intranucleolar condensations.

c. Nucleolus after 24 hours of infection. Hypertrophy and dense spots of increased density on nucleolonema.

d, e. Two rare types of lesions observed after 12 hours and 24 hours of infection.

f, g, h. Nucleoli observed 4 to 10 days after infection. *f*, large holes containing viruses; *g*, amorphous, atrophied nucleolus; *h*, clusters of coarse, dense granules within and at the periphery of a homogeneous nucleolar mass.

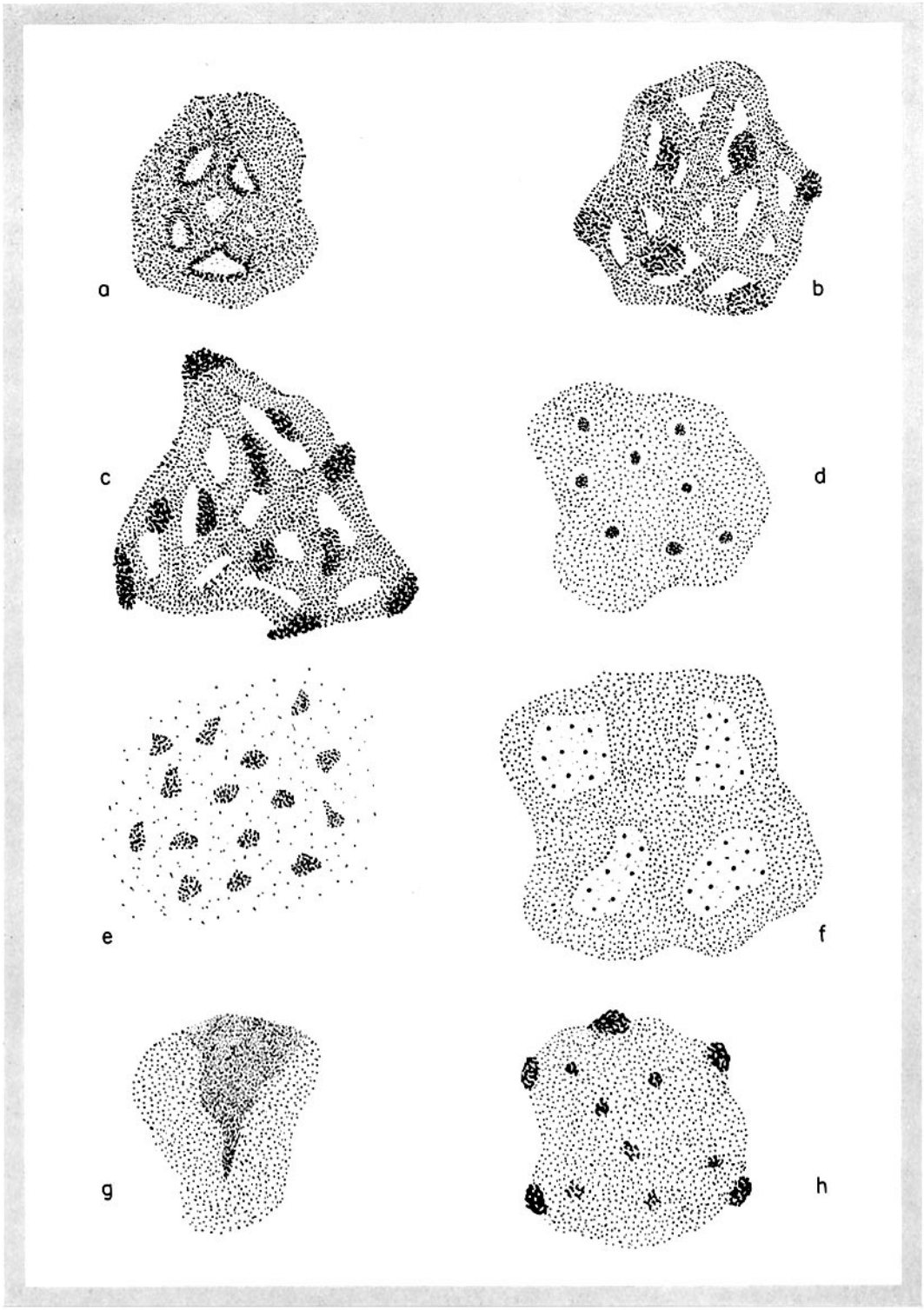


FIGURE 2

dense bodies of about 0.5 μ. They appear to be composed of thin filaments (Fig. 7). In the center of some of these bodies clusters of spherical granules having the density and the size of the SV40 virus are clearly visible (Fig. 8). The cytoplasmic vacuoles are increasingly numerous.

From the 3rd to the 7th day, the lesions described above are always present and are more frequent. The number of nuclei containing very numerous virus particles gradually increases. Moreover, in some of these nuclei, one or several spherical bodies with clusters of viruses are still present. In some cases such bodies, fully packed with viruses, are delimited by a thin outer envelope which, however, appears discontinuous at some places (Fig. 9). It seems probable that such spherical viral inclusions arise from the dense nuclear condensations already visible in the first stage of virus infection (Figs. 3 and 7). The same spherical bodies packed with virus particles have been described in some nuclei of mouse embryo cells infected with polyoma virus (3) (Fig. 10).

In addition, the tendency of the viruses to form crystalline aggregates is already evident. Such appearances cannot be distinguished from those observed in polyoma-infected nuclei.

In the cytoplasm the number and chiefly the size of the vacuoles increase considerably to give an electron microscopic picture of the cytopathic effect which corresponds to that described in light microscopy (38, 24, 15). Virus particles can be found in the cytoplasm in general after 6 days of infection, and in some cases earlier. However, the vacuolization of the cytoplasm cannot be fully explained by the presence of cytoplasmic virus. This lesion seems to be rather a consequence of the nuclear changes.

From this stage on up to the 10th or 11th day, during which the final lysis of infected cells occurred in our cell system, many nuclei appear packed with virus. A detailed study of the morphology of these viral particles and of the late cellular alterations linked with the viral multiplication is particularly easy in such advanced stages of infection.

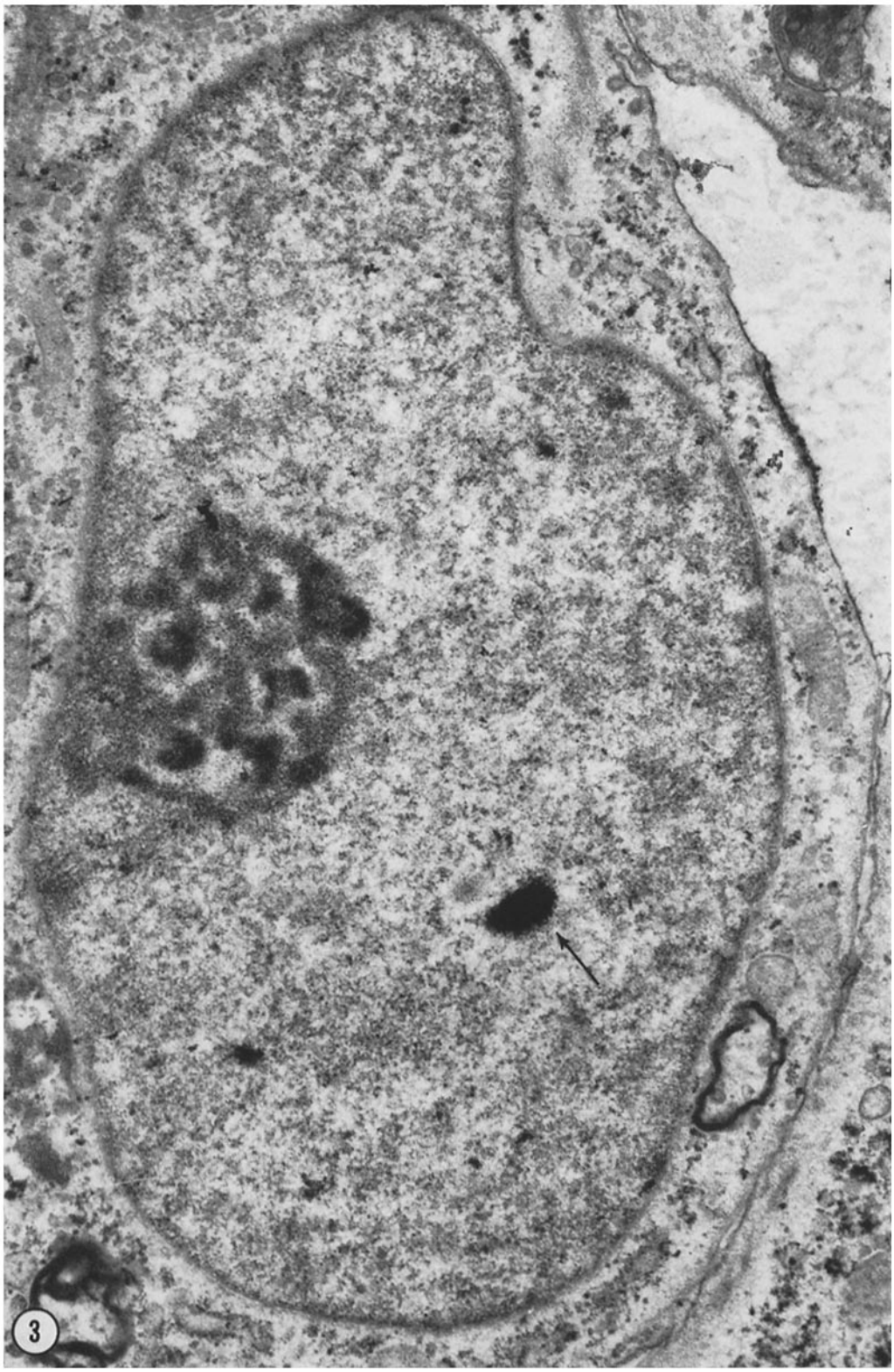
The medium diameter of the SV40 virus is 33 mμ, and the regularity of its size is striking. The viral particles seem to be spherical (Fig. 11). Their density depends on the electron stain used. It can be very much increased by double staining with uranyl acetate followed by lead acetate. The viruses do not possess an external membrane, but, at high magnification, a narrow outer zone may be distinguished around the central core or nucleoid which probably represents the capsid (Fig. 11). The value of 33 mμ refers to Epon embedding and double staining, which also increases the contrast of the capsid. But in spite of this, the exact limit of the particle is not always clearly visible. In these late stages the crystal-like arrangement of the virus is frequent. The center-to-center distance of the particles within the "crystal" is 40 to 42 mμ.

In addition to the presence of virus inclusions, several other cellular alterations are observed. In nuclei containing only a few viruses one observes margination of the chromatin clumped at the inner surface of the nuclear membrane (Fig. 12). However, in the majority of the nuclei the chromatin has disappeared almost totally and the few persistent fragments always show a very close association with virus particles.

Two other alterations are frequently found in infected nuclei: On the one hand, there are some clusters of coarse, extremely dense granules, very often in contact with the nucleolus (Fig. 12); on the other hand, one observes bundles of fibrils in virus-containing areas (Fig. 12). At high magnification, these fibrils do not show any periodic structure. They resemble the fibrillar structures found in nuclei of mouse cells infected with the thymic agent (4). One can find intermediate stages between these fibrillar bundles and well delimited light zones containing a loose, filamentous network, visible in between the virus crystals. However, it has not been possible to observe stages in the direct transformation of fibrillar bundles into such reticular areas, and the significance of this observation is still unknown. Three

FIGURE 3

Cercopithecus kidney cell, 10 hours after infection with SV40 virus. Nucleolus in close contact with nuclear membrane. The nucleolonema shows wider meshes than in control cells. Increased density of some distinct areas in the nucleolar substance. Arrow indicates dense body in nucleoplasm, of unknown nature. The cytoplasm appears normal. × 30,000.


3

types of nucleolar alterations are visible in these highly advanced and degenerative stages:

a) In hypertrophied nucleoli there frequently exist large holes containing chromatin-like material with numerous virus particles (Fig. 2, *f*).

b) Other nucleoli are composed of a homogeneous, finely granular material containing clusters of the coarse and very dense granules described above (Fig. 12). These clusters are mainly localized at the periphery of the nucleolus and seem to give rise to similar granules dispersed throughout the nucleoplasm.

c) Finally, in some nuclei totally filled with viruses there is a striking nucleolar atrophy and one observes the presence of an extremely dense material attached to the nucleolonema. This nucleolar lesion may be observed also in early stages of infection (Fig. 6). Finally the cytoplasm appears completely vacuolated, with formation of myelin figures. In addition, there are often many intracytoplasmic particles which either have been released from the nucleus after partial disruption of the nuclear membrane or have been phagocytized from the surrounding fluid. It is not unusual to find virus particles between the two sheets of the nuclear membrane. In the cytoplasm the viruses are most often attached to the membranes of vacuoles (Fig. 13). More rarely a small crystal-like cluster without a limiting membrane is found in the cytoplasm. In these two cases the viral particles have the same morphology as in the nucleus (Fig. 13). But there also exists another type of particle in the cytoplasm. This different type of particle is most often gathered into small groups. Its mean diameter is about 50 mμ, therefore much larger than that of intranuclear viruses. These large viral particles possess an external membrane (Fig. 13). A relationship may exist between the limiting membrane of the vacuoles and the formation of the external membrane of these large cytoplasmic viruses. Loose clusters of virus particles also have been found inside a zone of small vesicles and a filamentous network. The lesions are similar in appearance to those existing in polyoma virus–infected mouse embryo cells.

Numerous viral particles are often found outside the cells, attached to the cellular membrane. Finally, we have also found some phagocytized inclusions (phagosomes) with cell debris and numerous viral particles whose morphology is similar to that of the intranuclear SV40 virus.

Development of SV40 Virus in Baboon Kidney Cells

No early stages of infection have been examined. *After 7 and 9 days* of infection, very numerous viruses are found in some nuclei. They present the same morphology as in the *Cercopithecus* kidney cells. Their tendency to form crystal-like inclusions is strong. The presence of the spherical bodies in the nucleoplasm containing clusters of viruses is more frequently seen here than in *Cercopithecus* cells (Fig. 9). They present a filamentous, whorled structure. In one of these condensations we have found several hollow tubular structures whose diameter was less than that of the mature viral particles. However, tubules similar to those described in polyoma virus–infected cells have not been found in our thin sections, but were encountered repeatedly in SV40 virus preparations after negative stain (6).

The nucleolar lesions are of the same type and as frequent as in *Cercopithecus*. The above-described cytoplasmic forms of the SV40 virus also exist in the baboon cells. Therefore, from the morphological point of view, we have not noticed any difference in the evolution of this virus in the cells of two different origins.

DISCUSSION

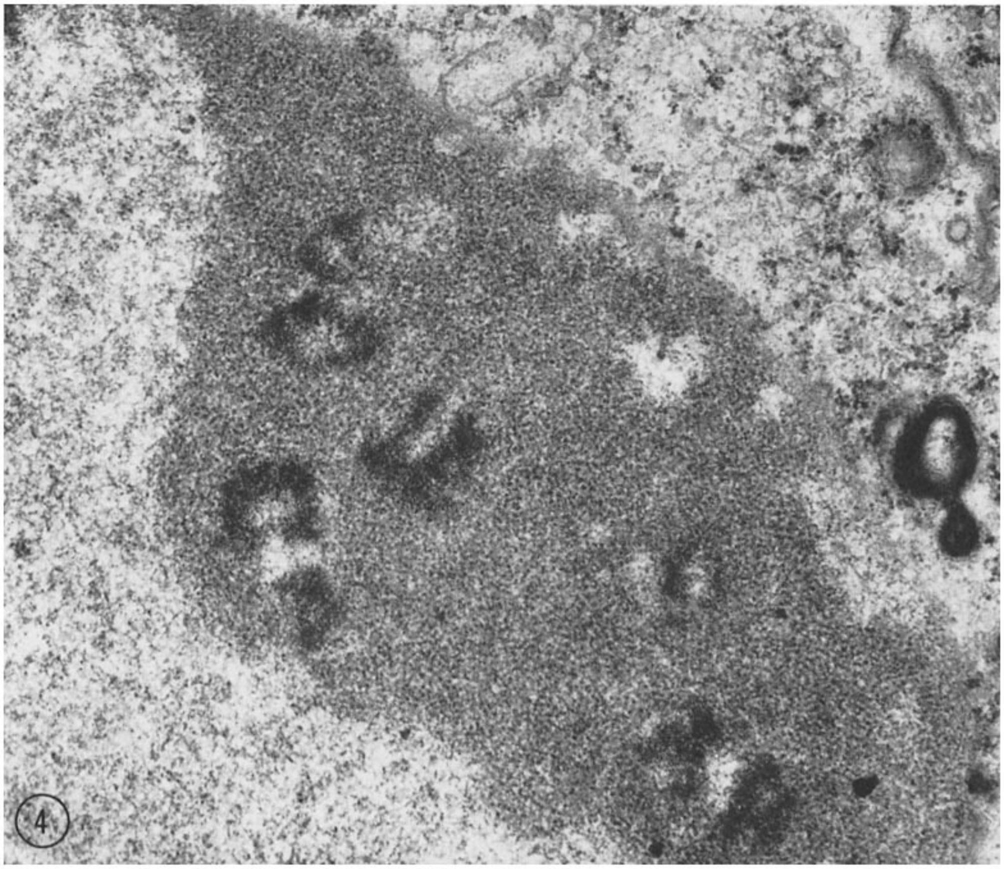
The data here presented entirely confirm our preliminary observations (40) and also those of Gaylord and Hsiung (15). The SV40 virus has essentially a nuclear development. The earliest

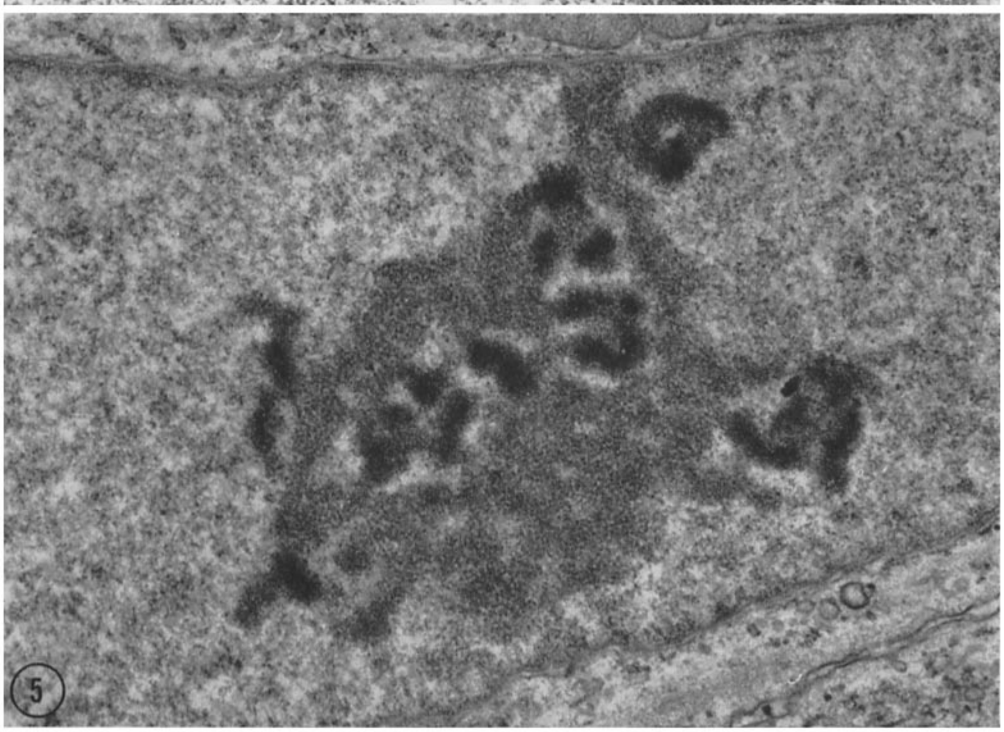
FIGURE 4

Cercopithecus kidney cell, 24 hours after infection. Hypertrophied and homogenized nucleolus with several dense horseshoe-like inclusions. ×30,000.

FIGURE 5

Cercopithecus kidney cell, 24 hours after infection. Hypertrophied nucleolus. Sausage-shaped condensations on the nucleolonema, some of which are visible at the periphery of the nucleolus. × 24,000.





lesions are found in the nucleolus and nucleoplasm. The later cytoplasmic lesions, particularly the appearance of vacuoles, are interpreted as the consequence of the nuclear alterations.

The presence of SV40 virus particles in a few nuclei as early as 10 to 12 hours after infection of the culture indicates that at least in some cells the development of the virus is much more rapid than was originally believed. In our preliminary study, we did not find virus particles before the 48th hour of infection (40). This was also the time of appearance of intranuclear lesions visible with the light microscope (15). Melnick (35) estimated the time of the infectious cycle as being 24 hours. We must emphasize that it is impossible to indicate the exact age of the lesions described above, as we certainly have in our system a superimposition of several infectious cycles after 2 or 3 days of culture. We could determine precisely the age of the observed lesions in terms of "hours after infection" only if we could be sure that the infection of all cells started simultaneously and that the speed of viral synthesis was the same in all cells; this is not the case here. All we can say is that in the majority of cases nuclear virus particles were seen only after 24 to 48 hours of infection, but in a very few cells they appeared much earlier.

The virus particle has a medium diameter of 33 mμ when measured on thin sections. This corresponds to the figure of 30 mμ ± 10 per cent given by Gaylord and Hsiung (15), but differs from that of 40 to 50 mμ published by Melnick (35). The latter author, however, does not indicate on which technique his measurement was based. If one measures the center-to-center distance of particles in a crystal on a section, one finds a diameter of 40 to 42 mμ; after negative staining, it is 42 to 45 mμ (6). If the shadow cast method were employed, we probably would obtain still higher values. In the latter two cases the particles are flattened after desiccation and, therefore, their diameter tends to increase. Apparently contradictory data found in the literature on the size of viruses usually can be explained by differences in technique. This is well illustrated by the related common wart virus, recently studied by Williams *et al.* (44).

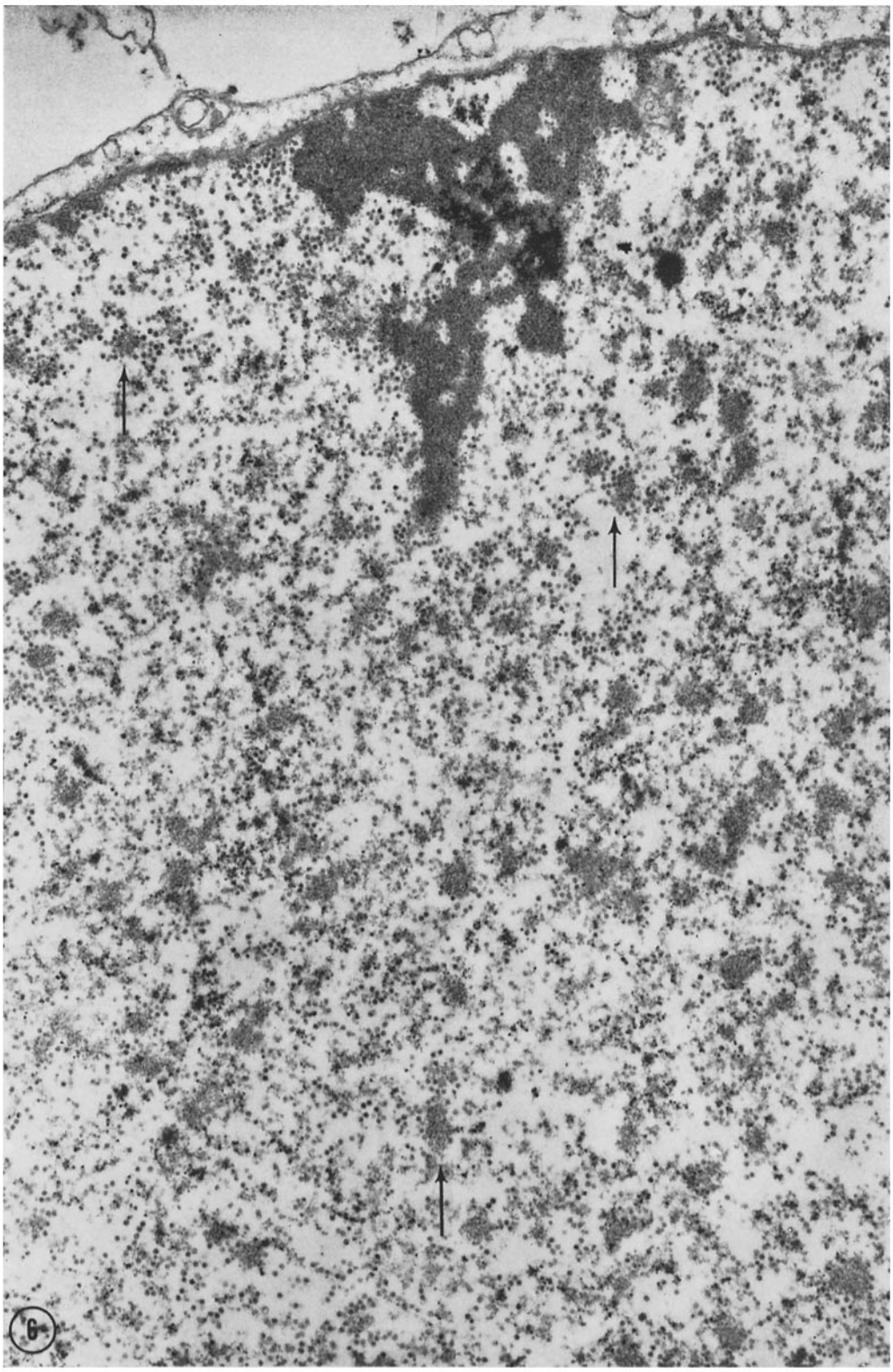
Morphologic studies reveal that the SV40 virus (15, 40) is strikingly similar to polyoma virus (2, 3, 22, 23) and also, though to a lesser extent, to the Shope papilloma (20, 37, 45) and human wart viruses (1, 9, 14, 44). They are each small, and in sections are seen to possess an outer zone of low density (the capsid), but no distinct peripheral membrane. They develop in the nucleus, where they have the tendency to form crystals. At least three of them are known to be DNA viruses: polyoma (34, 41), Shope papilloma (26), and SV40 (7, 16). Examination by the negative staining method, however, seems to reveal discrepancies. Despite the icosahedral symmetry which all exhibit, there is as yet no agreement on the number of capsomeres. The polyoma (43), human wart (44), and SV40 viruses (33, 6) seem to have 42 capsomeres, although this still needs confirmation. The number of capsomeres is not yet well established for the Shope papilloma virus: 42 or 60 (45), 60 to 70 (8). Moreover, a model with 92 units is shown by Mattern to be as probable as the 42-unit model for the polyoma and papilloma viruses (32). Until such time as agreement is reached on details of fine structure, it would seem premature to place these four viruses in the same group, as proposed by Melnick (Papova group (35)).

Although the nuclear lesions produced by polyoma and SV40 viruses are generally similar (3, 40), they do exhibit certain differences. Only the SV40 virus has been seen to induce the formation of bundles of fibrils. In the case of the Shope papilloma (37) and common wart (44) viruses there seems to be a closer topographical relationship between these agents and the nucleolus than in the other viruses, but this has to be investigated further.

The relationships of these viruses with the chromatin and the nucleolus merit special attention. Fragmentation and margination of the chromatin is a common feature of this type of

FIGURE 6

Cercopithecus kidney cell, 48 hours after infection. Appearance in the nucleus of numerous virus particles which are seen in close contact with the small clumps of chromatin (arrows) disseminated throughout the nucleoplasm. Atrophied nucleolus. In contact with the nucleolonema, very dense granular substance of unknown significance. × 20,000.



infection. The role of the chromatin in the formation of polyoma (22) and SV40 virus seems important. Gaylord and Hsiung (15) have even hypothesized that the chromatin of SV40-infected cells is somehow transformed into virus particles. In the case of polyoma virus, we have noticed that the virus particles are frequently situated in the peripheral region of the nucleoplasm close to the nuclear membrane. Though it is less common, we have also observed the same phenomena for the SV40 virus after formalin or acrolein fixation followed by embedding in a water-soluble plastic as proposed by Leduc *et al.* (29) and applied to the study of virus infection by Bernhard and Tournier (5). Virus particles gradually seem to replace the chromatin, and in the early stage of their appearance they are in close contact with the chromatin clumps, but not with the interchromatinic proteins. The particles appear simultaneously throughout the chromatin, wherever it is located, mainly along the nuclear membrane, or in contact with the nucleolus-associated chromatin and, therefore, close to the nucleolus, but not in the nucleolar substance itself. The observation made by Stone *et al.* (37) on the appearance of Shope papilloma virus within the nucleolus and a similar finding reported by Almeida *et al.* (1) in the case of common wart virus has not been observed in the case of polyoma and SV40 virus. Indeed, the presence of SV40 virus inside the large holes which appear in the nucleolar mass after several days of infection does not mean that these viruses have been formed in the nucleolus. This may correspond as well to the penetration of the nuclear substance into the nucleolus during the uncoiling of the nucleolonema, a phenomenon we have frequently observed (Fig. 2, *f*).

Therefore we agree with Gaylord and Hsiung (15) that the first virus particles, as a rule, do not appear in the nucleolus.

There is no doubt, however, that the nucleolus is somehow actively involved in the process of viral synthesis. The hypertrophy of this organelle in the late eclipse phase and the appearance of a dense, newly formed substance in the nucleolonema are striking in both polyoma and SV40 infection. One can consider this early nucleolar alteration to be the morphological expression of a changed nucleolar function in relation to the infectious process. Such nucleolar lesions have been noticed in infections with other groups of viruses, thus indicating that this phenomenon probably has a more general significance than has been heretofore appreciated. Hypertrophied and "spotted" nucleoli have been described in varicella (39), ectromelia (28), mouse encephalomyocarditis (21), molluscum contagiosum (10), Rous sarcoma (19), and mouse leukemia (18). The chemical nature of these dense condensations is not yet known because they are in general at the limit or beyond the resolution of the light microscope and, therefore, inaccessible to the usual cytochemical investigation. An interesting light microscopic attempt to analyze the chemical composition of the enlarged nucleolus in polyoma virus infection has been made by Love and Rabson (30), but it is not clear which structures visible in the electron micrographs correspond to the RNA-containing nucleolini described by these authors. We have found by methods of ultrastructural cytochemistry that intranucleolar chromatin, rare in normal controls, is considerably increased in the nucleoli of SV40-infected cells. This chromatin was arranged sometimes in regularly spaced bands, alternating with the nucleolar substance, a phenomenon which was never seen in controls (5). The relationship between nucleolus-associated

FIGURES 7 AND 8

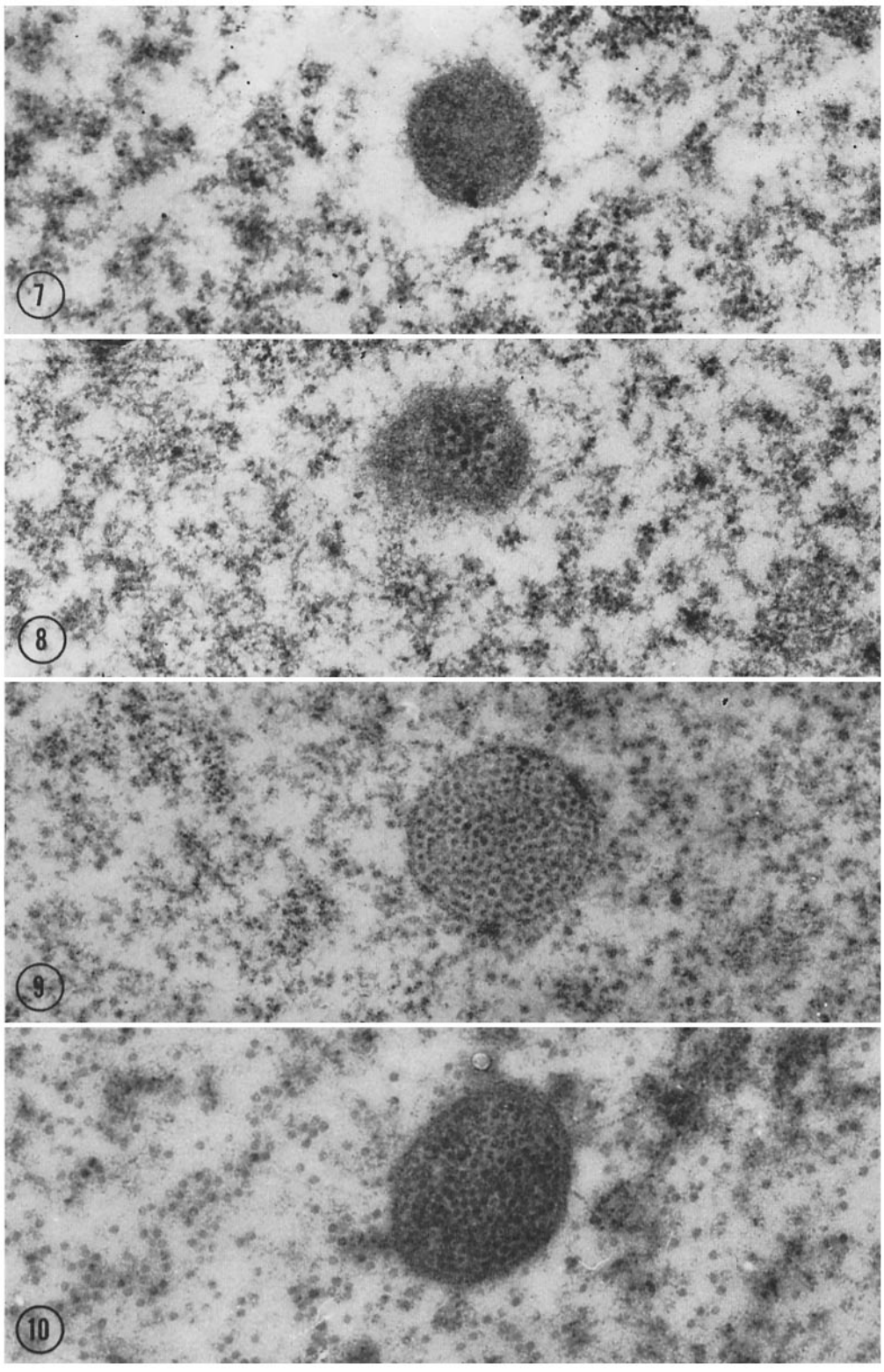
Cercopithecus kidney cell, 48 hours after infection with SV40 virus. Small area of two nuclei with dense, spherical bodies. In Fig. 8, appearance of a few virus particles in the center of the body. × 42,000.

FIGURE 9

Baboon kidney cell, 7 days after infection with SV40 virus. Spherical body in nucleus entirely filled with virus particles, and delimited by a discontinuous envelope. × 42,000.

FIGURE 10

Embryonic mouse fibroblast, 3 days after infection with polyoma virus. Area of a nucleus with similar spherical body containing polyoma virus. × 42,000.


7
8
9
10

chromatin, nucleolar substances, particularly the RNA, and the synthesis of both viral DNA and specific proteins will have to be studied further. The very early reaction of the nucleolus in the course of the virus infection here described would suggest that some important mechanism of synthesis takes place in this organelle.

Finally, as far as cytoplasmic virus particles are concerned, they have been found in both SV40 and polyoma infection and give rise to similar pictures: In both cases some cytoplasmic viruses were observed with a diameter larger than that of the nuclear viruses: 50 mμ for the SV40, and 50 to 60 mμ for the polyoma agent (11, 22). These larger particles appear to have an outer membrane. It was thought that this membrane is acquired in the process of phagocytosis of extracellular particles (11). But in the case of SV40 virus described here, phagocytized particles, at least those that are present in great number in large vacuoles, do not exhibit a viral membrane. One also can hypothesize that some virus particles are coated with an envelope during their individual passage across the nuclear membrane. As we have mentioned above, we have indeed found virus particles, but without membrane, between the two leaflets of the nuclear membrane. It seems improbable that the viral membrane is a defense reaction of the cytoplasm toward the infection, a possibility mentioned by Howatson and Almeida (22). We must admit that the significance of the large cytoplasmic particle is not yet entirely understood.

The cytoplasmic vacuoles are considered to be a secondary reaction. They appear only in cells with severe nuclear damage. These vacuoles are very frequently associated with cytoplasmic virus. The particles are found mostly between the vacuoles, attached to the outer surface of their membrane.

In conclusion, we can say that the SV40 virus has, in all respects, a great morphological similarity to the polyoma virus. This morphological similarity is less evident for the Shope papilloma and the common wart virus, whose intracellular evolution has still to be studied further. However, their similar morphological features can be placed in line with their similar biological properties, in particular their oncogenicity. Three of these viruses give rise to malignant tumors.

One may wonder if the human wart virus might not also induce malignant transformation in a suitable system. The morphological criteria are now sufficiently known to be used as a basis for further biological studies on these viruses or related agents.

This work was aided by grant C-4602 (C3) from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and by the Mutuelle Générale de l'Éducation Nationale, Paris, France.

We gratefully acknowledge the excellent technical assistance of Mlle Simone Paillet.

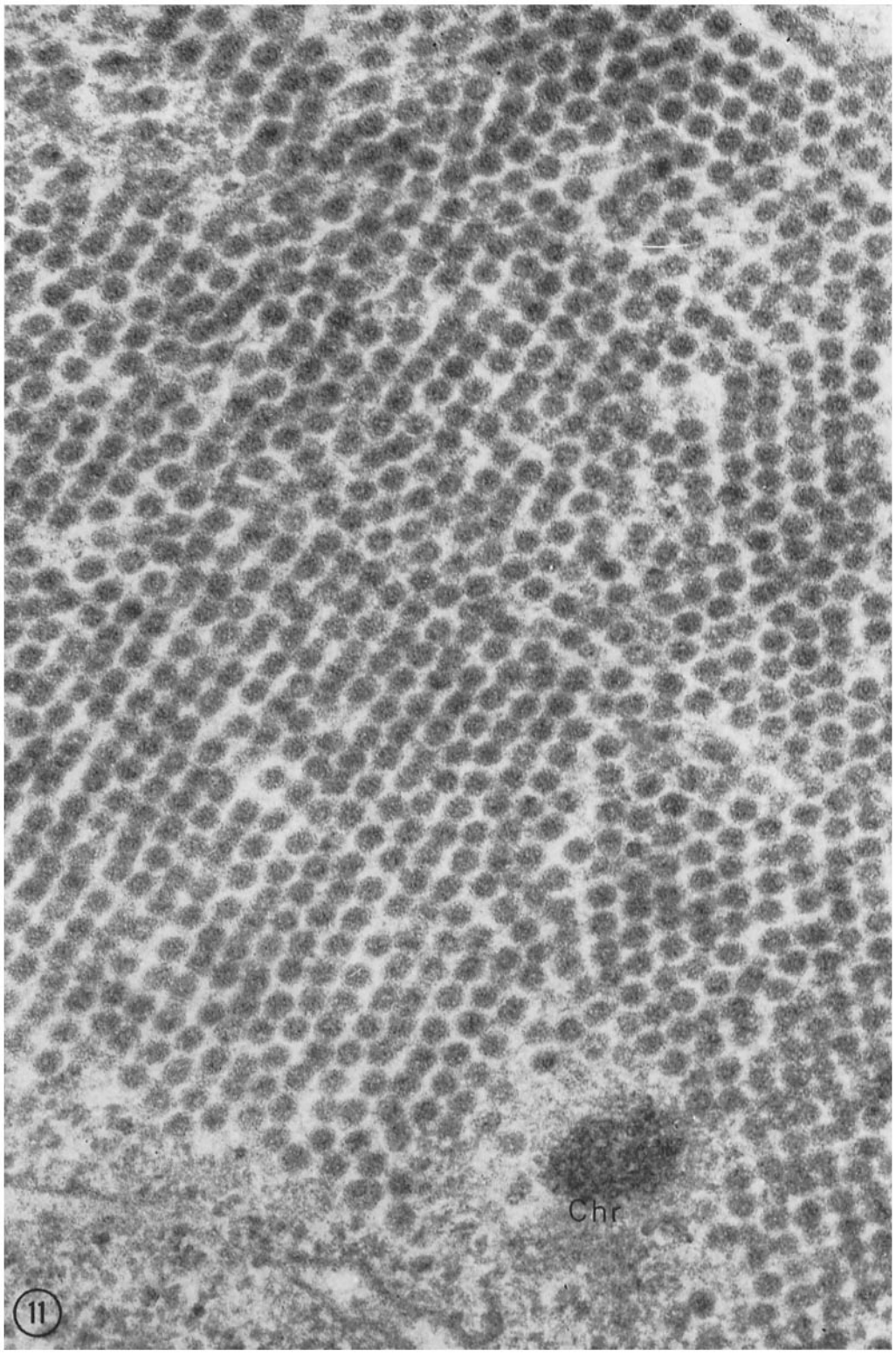
Received for publication, October 15, 1962.

REFERENCES

1. ALMEIDA, J. D., HOWATSON, A. F., and WILLIAMS, M. G., Electron microscope study of human warts, sites of virus production and nature of the inclusion bodies, *J. Inv. Dermat.*, 1962, **38**, 337.
2. BANFIELD, W. G., DAWE, C. J., and BRINDLEY, D. C., Intracellular particles in tissue cultures inoculated with parotid-tumor-agent (Polyoma virus), *J. Nat. Cancer Inst.*, 1959, **23**, 1123.
3. BERNHARD, W., FEBVRE, H. L., and CRAMER, R., Mise en évidence au microscope électronique d'un virus dans des cellules infectées *in vitro* par l'agent du polyome, *Compt. rend. Acad. sc.*, 1959, **249**, 483.
4. BERNHARD, W., and GRANBOULAN, N., Morphology of oncogenic and non oncogenic mouse viruses, *in* Ciba Symposium on Tumor Viruses of Murine Origin, London, Churchill Ltd., 1962, 6.
5. BERNHARD, W., and TOURNIER, P., Ultrastructural cytochemistry applied to the study of virus infection, *Cold Spring Harbor Symp. Quant. Biol.*, 1962, **27**, 67.
6. BERNHARD, W., VASQUEZ, C., and TOURNIER, P.,

FIGURE 11

Cercopithecus kidney cell, 10 days after infection with SV40 virus. Higher magnification of virus particles in a nucleus. Around the dense nucleoids, an outer zone (capsid) with less contrast is clearly visible in some particles. *Chr*, chromatin. × 120,000.

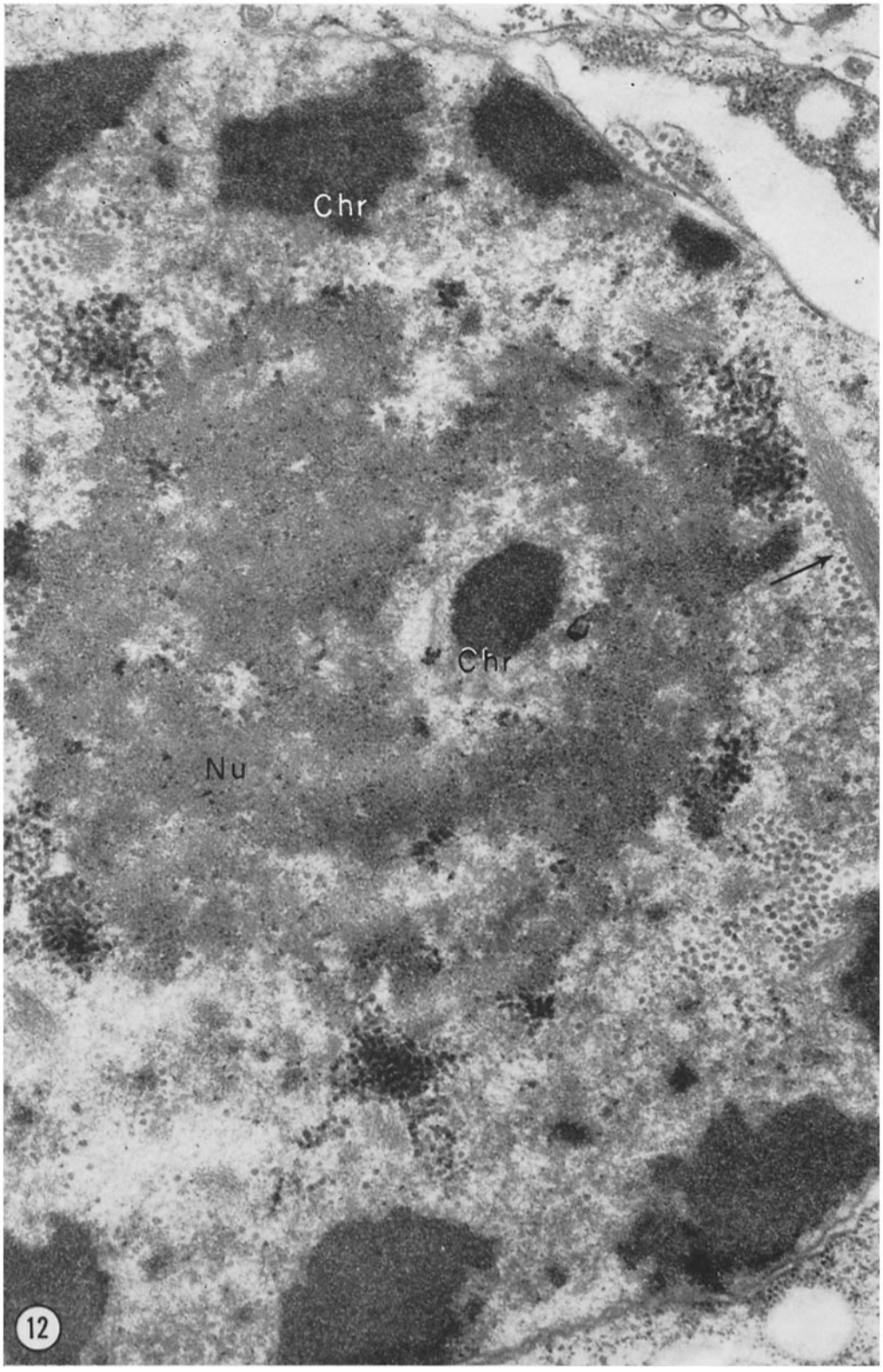

Chr
11

La structure du virus SV40 étudiée par coloration négative au microscope électronique, *J. micr.*, 1962, **1**, 331.

7. BOIRON, M., PAOLETTI, C., THOMAS, M., REBIERE, J. P., and BERNARD, J., Acide désoxy-ribonucléique infectieux extrait de cultures de cellules de rein de singe babouin infectées par le virus SV40, *Compt. rend. Acad. sc.*, 1962, **254**, 2097.
8. BREEDIS, C., BERWICK, L., and ANDERSON, T. F., Fractionation of Shope papilloma virus in cesium chloride density gradients, *Virology*, 1962, **17**, 84.
9. CHARLES, A., Electron microscope observations of the human wart, *Dermatologia*, 1960, **121**, 193.
10. DOURMASHKIN, R., and BERNHARD, W., A study with the electron microscope of the skin tumor of molluscum contagiosum, *J. Ultrastruct. Research*, 1959, **38**, 11.
11. DOURMASHKIN, R., and NEGRONI, G., The cytoplasmic phase of the life cycle of polyoma virus in mouse embryo cell cultures, *in Proc. European Regional Conf. Electron Micr.* (Delft, 1960), **2**, 978.
12. DULBECCO, R., and FREEMAN, G., Plaque production by the polyoma virus, *Virology*, 1959, **8**, 396.
13. EDDY, B. E., BORMAN G. S., BERKELEY, W. H., and YOUNG, R. D., Tumors induced in hamsters by injection of rhesus monkey kidney cell extracts, *Proc. Soc. Exp. Biol. and Med.*, 1961, **107**, 191.
14. GAYLORD, W. H., Cellular reaction during virus infections, *in* Frontiers in Cytology, (S. L. Palay, editor), New Haven, Yale University Press, 1958, 447.
15. GAYLORD, W. H., JR., and HSIUNG, G. D., The vacuolating virus of monkeys. II. Virus morphology and intranuclear distribution with some histochemical observations, *J. Exp. Med.*, 1961, **114**, 987.
16. GERBER, P., An infectious deoxyribonucleic acid derived from vacuolating virus (SV40), *Virology*, 1962, **16**, 96.
17. GIRARDI, A. J., SWEET, B. H., SLOTNICK, V. B., and HILLEMAN, M. R., Development of tumors in hamsters inoculated in the neonatal period with vacuolating virus SV40, *Proc. Soc. Exp. Biol. and Med.*, 1962, **109**, 649.
18. GRANBOULAN, N., and RIVIERE, M. R., Étude au microscope électronique des particules virales présentes dans des lymphomatoses spontanées de la souris, *J. micr.*, 1962, **1**, 23.
19. HAGUENAU, F., Significance of ultrastructure in virus induced tumors, *in* Symposia Tumor Viruses, *Nat. Cancer Inst. Monograph No. 4*, 1960, 211.
20. HAGUENAU, F., BONAR, R. A., BEARD, D., and BEARD, J. W., Ultrastructure of the rabbit papilloma virus, *J. Nat. Cancer Inst.*, 1960, **24**, 873.
21. HINZ, R. N., BARSKI, G., and BERNHARD, W., An electron microscopic study of the development of encephalomyocardite (EMC) virus propagated *in vitro*, *Exp. Cell Research*, 1962, **26**, 571.
22. HOWATSON, A. F., and ALMEIDA, J. D., An electron microscope study of polyoma virus in hamster kidney, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 753.
23. HOWATSON, A. F., and ALMEIDA, J. D., Observations on the fine structure of polyoma virus, *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 828.
24. HSIUNG, G. D., and GAYLORD, W. H., The vacuolating virus of monkeys. I. Isolation, growth characteristics, and inclusion body formation, *J. Exp. Med.*, 1961, **114**, 975.
25. HUXLEY, H. E., and ZUBAY, G., Preferential staining of nucleic acid-containing structures for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 273.
26. ITO, Y., and EVANS, C. A., Induction of tumors in domestic rabbits with nucleic acid preparations from partially purified Shope papilloma virus and from extracts of the papillomas of domestic and cottontail rabbits, *J. Exp. Med.*, 1961, **114**, 485.
27. KARNOVSKY, M. J., Simple methods for "staining with lead" at high pH in electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 729.
28. LEDUC, E. H., and BERNHARD, W., Electron microscope study of mouse liver infected by ectromelia virus, *J. Ultrastruct. Research*, 1962, **6**, 466.
29. LEDUC, E. H., MARINOZZI, V., and BERNHARD, W., The use of water-soluble glycolmethacrylate in ultrastructural cytochemistry, *in* Oxford

FIGURE 12

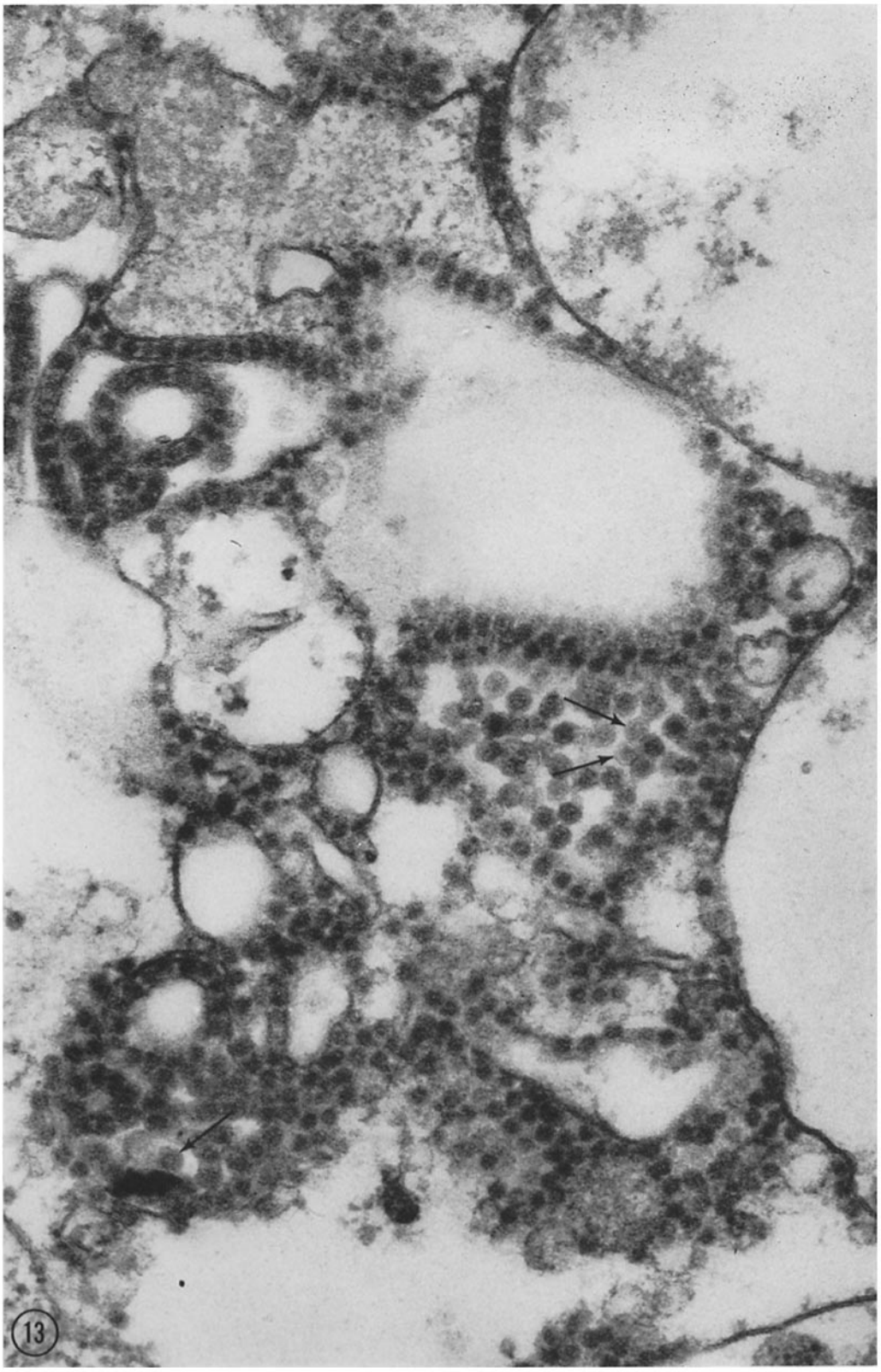
Cercopithecus kidney cell, 10 days after infection with SV40 virus. Nucleus showing margination of chromatin (*Chr*). Nucleolus (*Nu*) composed of a finely granular substance of low contrast. In its center is chromatin. At its periphery, clusters of coarse, very dense granules. A few virus particles visible in the nucleoplasm. Arrow indicates bundle of fibrils of unknown significance. × 36,500.


Chr
Chr
Nu
12

Symposium on Cytochemical Progress in Electron Microscopy, *J. Roy. Micr. Soc.*, Suppl., 1962, **81**, 119.
30. Love, R., and Rabson, A. S., Nucleoproteins in murine lymphoma cells infected with polyoma virus "in vitro," *Path. et Biol.*, 1961, **9**, 694.
31. Marinozzi, V., and Gautier, A., Étude des affinités des composants nucléoprotéiniques pour l'hydroxyde de plomb et l'acétate d'uranyle, *J. Ultrastruct. Research*, 1962, **7**, 436.
32. Mattern, C. F. T., Polyoma and papilloma viruses: Do they have 42 or 92 subunits?, *Science*, 1962, **137**, 612.
33. Mayor, H. D., and Melnick, J. L., Icosahedral models and viruses: A critical evaluation, *Science*, 1962, **137**, 613.
34. Di Mayorca, G. A., Eddy, B. E., Stewart, S. E., Hunter, W. S., Friend, C., and Bendich, A., Isolation of infectious deoxyribonucleic acid from SE polyoma infected tissue cultures, *Proc. Nat. Acad. Sc.*, 1959, **45**, 1805.
35. Melnick, J. L., Papova virus group, *Science*, 1962, **135**, 1128.
36. Millonig, G., Advantages of a phosphate buffer for OsO_4 solutions in fixation, *J. Appl. Physics*, 1961, **32**, 1637.
37. Stone, R. S., Shope, R. E., and Moore, D. H., Electron microscope study of the development of the papilloma virus in the skin of the rabbit, *J. Exp. Med.*, 1959, **110**, 543.
38. Sweet, B. H., and Hilleman, M. R., The vacuolating virus SV40, *Proc. Soc. Exp. Biol. and Med.*, 1960, **105**, 420.
39. Tournier, P., Cathala, F., and Bernhard, W., Ultrastructure et développement intracellulaire du virus de la varicelle, *Presse Méd.*, 1957, 1230.
40. Tournier, P., Granboulan, N., and Bernhard, W., Examen au microscope électronique de cellules de rein de Cercopithèque infectées in vitro par le virus SV40, *Compt. rend. Acad. sc.*, 1961, **253**, 2283.
41. Weil, R., A quantitative assay for a subviral infective agent related to polyoma virus, *Virology*, 1961, **14**, 46.
42. Wicker, R., and Tournier, P., unpublished data.
43. Wildy, P., Stocker, M. G. P., MacPherson, I. A., and Horne, R. W., The fine structure of polyoma virus, *Virology*, 1960, **11**, 444.
44. Williams, M. G., Howatson, H. F., and Almeida, J. D., Morphological characterization of the virus of the human common wart (verruca vulgaris), *Nature*, 1961, **189**, 895.
45. Williams, R. C., Kass, S. J., and Knight, C. A., Structure of Shope papilloma virus particles, *Virology*, 1960, **12**, 48.

Figure 13

Cercopithecus kidney cell, 10 days after infection. Virus particles in between cytoplasmic vacuoles. Most particles have the same morphology as those described in the nucleus, but a few are larger (50 mμ) and it may be possible to distinguish an outer membrane in some of them (arrows). × 80,000.


13