INTRACELLULAR LOCALIZATION OF TYPE 4 ADENOVIRUS

II. Cytological and Fluorescein-Labelled Antibody Studies*

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Plates 10 to 12

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The cytologic alterations which develop in HeLa cells infected with adenoviruses have been described by a number of investigators using both light and electronmicroscopy (1–6). The changes observed, including the formation of Feulgen-positive intranuclear crystals composed of virus-like particles, suggest that the nucleus is the major site of adenovirus synthesis. Such a conclusion is of course justified only if the arrays of virus-like particles found in infected cells are actually aggregates of virus. As discussed in the preceding paper, the results of HeLa cell fractionation studies indicated that infectious type 4 adenovirus was associated with the "cytoplasmic" rather than the "nuclear" fraction, a finding which raised some question about the interpretation of the intranuclear changes (7). When the results of the fractionation studies were obtained, several possibilities concerning the nature of the intranuclear changes were considered. One was that the particles observed by electron microscopy were infectious virus, but because of the tightly packed crystalline arrays, each aggregate was measured as an infectious unit and the properties of the individual particles could not exert themselves. Other possibilities were: that the regularly aligned particles in the crystalline masses in infected nuclei were composed principally of non-infectious virus; that intranuclear alterations were the result of deranged host cell metabolism not directly associated with viral synthesis; and that the changes were terminal by-products of host cell-virus interaction such as the eosinophilic type A inclusion bodies seen late in the course of herpes simplex infection (8, 9).

The fluorescein-labelled antibody technique (10), by which intracellular viral antigen could be localized, provided a means of gaining further informa-

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tion on the nature of the cytologic alterations as well as a means of detecting viral antigen which was not associated with recognized morphologic change. This method was therefore employed in a sequential study of HeLa cells infected with type 4 adenovirus and the results were compared with observations of companion cultures by light and phase-contrast microscopy. Since the fluorescein-labelled antibody technique could furnish information on the antigenicity, but not on the infectiousness of the cell structures under study, additional experiments were performed to correlate the microscopic changes with the production of infectious virus.

Materials and Methods

Virus.—The type 4 adenovirus (RI 67 agent) isolated by Hilleman and Werner (11) was used; it had been propagated by passage in HeLa cells as reported previously (12).

Tissue Culture.—Cells of the HeLa strain (13) were propagated and maintained in bottles and in culture tubes as described previously (12, 14). For cytological studies the cells were grown on strips of coverglass placed in 16 x 150 mm. screw cap tubes. Companion cultures employed in virus growth curve titrations were made in tubes lacking coverslips; the handling of the cultures was otherwise identical.

Infection of Tissue Culture Tubes.—Cultures were inoculated with 0.1 ml. of a 10^{-2} dilution of stock virus, containing $10^{1.5}$ to $10^{3.0}$ infectious doses; more concentrated virus suspensions produced undesirable "toxic" effects (6, 15). After a period of 6 hours to allow viral adsorption, the virus-containing fluid was removed, and in the case of the coverslip cultures was replaced immediately with 1 ml. of fresh maintenance fluid. In some experiments horse serum (10 per cent of final volume) was incorporated into the maintenance medium in an attempt to decrease the rounding of cells in infected cultures (16). The tube cultures used in the study of the production of infectious virus were washed with 4 ml. of Hanks' solution to remove residual unadsorbed virus before maintenance mixture was added. At intervals varying from 6 to 72 hours after infection, one set of infected cultures was harvested for virus titration and a companion set of coverslip cultures was prepared for microscopic examination.

Infectivity Titrations.—The methods used are described in a preceding paper (12).

Preparation of Cells for Microscopic Study.—Cultures for light microscopic study were fixed with 95 per cent ethyl alcohol and stained with hematoxylin and eosin (6). For phase microscopic observations coverslip cultures were withdrawn from the tubes, inverted on clean glass slides in a few drops of culture medium and their edges sealed with warmed paraffin-permount mixture. Such cultures were examined immediately under the phase-contrast microscope (Zeiss; positive phase-contrast system).

Staining for Adenovirus Antigens with Fluorescent Antibody.—The indirect fluorescent antibody technique described by Weller and Coons (17) was used for the visualization of viral antigens. The coverslip cultures were washed in 0.85 per cent NaCl solution buffered with 0.01 M phosphate at pH 7.2, hereafter termed buffered saline solution, and dried for 1 hour at 37°C. The cells were then fixed for 10 minutes in acetone and dried for 30 minutes at 37°C. Immune rabbit antiserum (18) diluted 1:10 with buffered saline was layered over the fixed cells for 30 minutes. The cultures were next washed for 10 minutes with buffered saline and then covered for 30 minutes with goat globulin containing fluorescein-labelled antibodies directed against rabbit gamma globulin.[1]

After a 10 minute wash in several changes of buffered saline the preparations were mounted

[1] The fluorescein-labelled antiserum was generously provided by Dr. A. H. Coons and Dr. Barbara Watson, Harvard Medical School, Boston.

in buffered glycerine (pH 7.0) and examined under the fluorescence microscope. The specificity of the fluorescent staining for type 4 adenovirus antigen was established by adding the fluorescein-labelled goat antiserum to the following controls:

1. Uninfected cultures treated with specific type 4 rabbit antiserum.
2. Type 4 adenovirus-infected cultures exposed to rabbit antisera to heterologous adenovirus types 1, 3, and 7.
3. Type 4 infected cultures treated with rabbit antiserum to uninfected HeLa cell components, prepared in the same manner as antisera to the adenoviruses.
4. Type 4 infected cultures overlaid with non-immune rabbit serum.
5. Cultures infected with other adenovirus types (1 and 3) and treated with rabbit antiserum to adenovirus type 4.

None of the above controls showed the bright fluorescence characteristic of antigen accumulations present in type 4 adenovirus–infected preparations treated with specific antibody to adenovirus type 4. Non-specific fluorescence, usually of much less intensity, was, however, observed in all controls, particularly in the cytoplasm and sometimes in what appeared to be nucleoli. At least 2 control preparations were therefore made as companions to each set of infected cultures studied in order that the distribution and intensity of specific staining could be better judged.

Fluorescence Microscope.—An AH-6 water-cooled mercury vapor lamp[2] served as the light source. Source filters included a 2 cm. pathway of 25 per cent $CuSO_4$, three one-half thickness 5840 Corning glassfilters, and a 3 mm. pathway of 9863 Corning glass. A standard monocular laboratory microscope with glass optics was used with a double thickness of Wratten gelatin filter 2-B for an ocular filter.

Fluorescence Photomicrography.—All photomicrographs were taken with a 35 mm. Leica 2F camera,[3] and panatomic-X film.[4] The exposure time was 4 to 8 minutes.

EXPERIMENTAL RESULTS

Light, Phase-Contrast, and Fluorescence Microscopic Observations of HeLa Cells Infected with Type 4 Adenovirus.—The development of characteristic intranuclear changes in tissue culture cells as a result of adenovirus infection has been described by a number of investigators (1–6). In an earlier report from this laboratory the sequential cytologic and cytochemical alterations produced in HeLa cells by type 4 adenovirus were presented in detail (6). The changes visible in infected cells stained with hematoxylin and eosin will be repeated below for the purpose of comparison with the appearance of specific viral antigen in cultures examined by fluorescence microscopy, and also with the sequential changes observed in fresh unfixed cells by phase-contrast microscopy. As in the previous publication the cytologic changes have been divided into 3 consecutive stages: the first stage predominated between 14 and 24 hours after infection, the second between 30 and 38, and the third between 48 and 72.

Stage 1, 14 to 24 Hours after Infection.—Characteristic changes within infected HeLa cell nuclei could be detected by all 3 microscopic means during this period.

[2] General Electric Company, Cleveland.
[3] Ernest Leitz, manufacturer, New York.
[4] Eastman Kodak Company, Rochester, New York.

In hematoxylin and eosin–stained cultures the first changes observed consisted of the development of small eosinophilic masses within the nuclei. Later during stage 1 the normal chromatin material was replaced by a mass of granules, often arranged in a fine meshwork, and the peripheral nuclear zones showed clearing (see Fig. 1).

The corresponding nuclear changes were readily seen in unfixed cultures examined by phase-contrast microscopy. Fine granules, sometimes arranged in a coarse network, were visible in stage 1 nuclei (Fig. 5). With beginning transition into stage 2 the granules became larger, and tiny, sharp-edged crystals[5] appeared among them and in the clear peripheral zone of the nucleus. The fine crystals were not well visualized in the hematoxylin and eosin preparations, probably as a result of the fixation and staining process (6, 19).

In cultures stained for viral antigen the specific fluorescent staining was confined to the nuclei of affected cells. The distribution of the specific fluorescence matched exactly that of the regularly aligned granules and small crystals observed in companion cultures by light and phase-contrast microscopy (compare Fig. 11 with Figs. 1 and 5). Aggregates of antigen corresponding to the early eosinophilic masses were not found.

Stage 2, 30 to 38 Hours after Infection.—In hematoxylin and eosin-stained cells the onset of stage 2 was marked by an increase in width of the clear peripheral nuclear zone and a change in the appearance of the central zone (Fig. 2). In some cells the central area resembled a coarse honeycomb and in others a mass of large granules of varying size. Homogeneously stained basophilic crystals were sometimes visible in the nuclear periphery and in the compartments of the honeycombed centers.

The appearance of unfixed cultures examined by phase microscopy demonstrated again the extent of the shrinkage and distortion of the crystals which had occurred in cultures stained with hematoxylin and eosin. The crystals were far more numerous and better preserved in unfixed cells. The transition into stage 2 in the unfixed cells was characterized by increasing size and prominence of the crystals. The crystalline masses were sometimes scattered throughout the nucleus (Fig. 6), or in what appeared to be more advanced stages of infection, concentrated in the nuclear periphery (Figs. 7 and 8). The nuclear membrane, though convoluted and distorted, could still be discerned in the majority of the cells in stage 2.

In the fluorescent antibody preparations the antigen distribution coincided with that of the crystals and the granular aggregates. In early stage 2 cells the small crystals were dispersed throughout the nucleus among the granules (see Fig. 12). In later stages the crystals containing viral antigen were clustered at the edges of the nucleus (see Fig. 13). The nuclear membrane was not stained by the fluorescent dye, and its position could only be inferred by comparison with cells examined by phase or light microscopy. Familarity with the cytopathology of adenovirus-infected cells was essential in interpreting the location of fluorescent staining in cells in both stage 2 and stage 3. The round central mass of a stage 2 or 3 nucleus might easily be mistaken for a whole nucleus and the surrounding crystalline masses as accumulations of antigen in the cytoplasm (see Figs. 14 and 15). An occasional affected cell did reveal

[5] The crystalline nature of these inclusions produced by adenoviruses in HeLa cells has been demonstrated by analysis of the packing structure of the virus-like particles which compose the inclusions (20).

brightly fluorescent granules lying in an indentation of the nucleus. The majority of the staining, however, was still intranuclear.

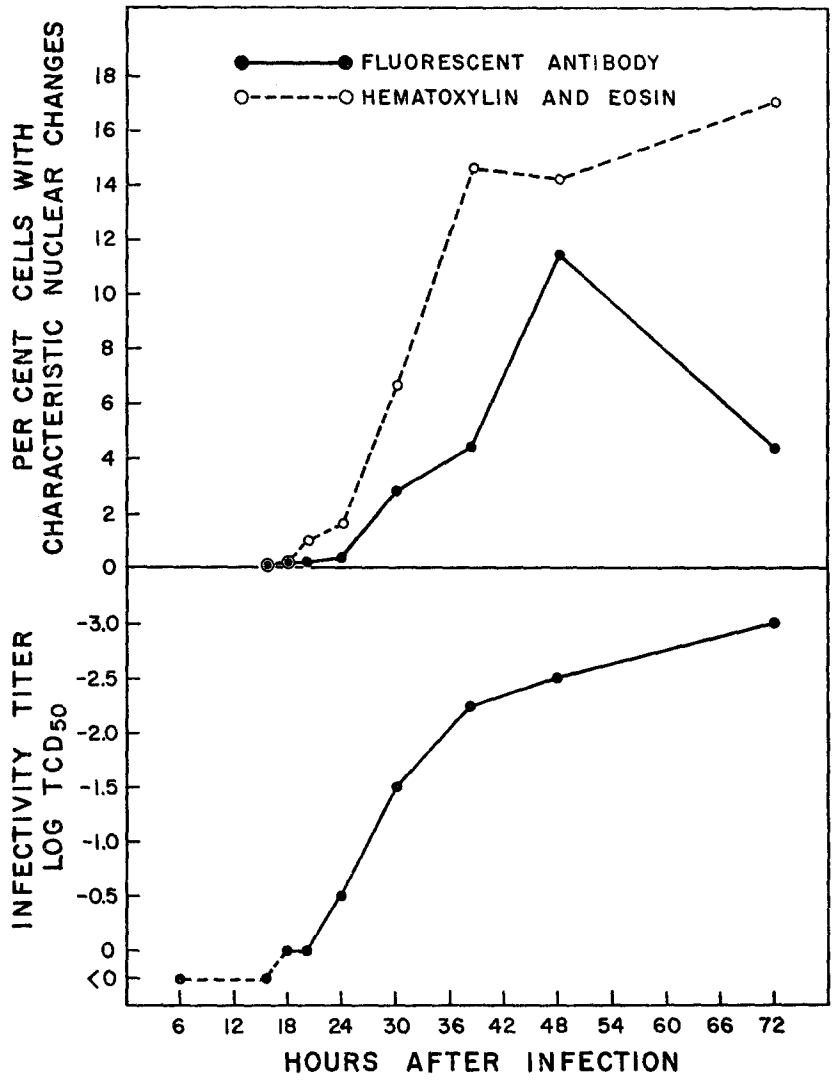
Stage 3, 48 to 72 Hours after Infection.—During this period, stage 3 cells became the dominant infected cell type, although earlier stages were also present. In hematoxylin and eosin preparations flower-like or rosette forms were frequent, with their intensely stained central masses and surrounding wedge-shaped or bubble-like compartments (Figs. 3 and 4). The peripheral nuclear zones of many nuclei contained large, basophilic crystals, whereas the peripheral zones of others contained amorphous homogeneously stained matter instead.

As in earlier stages of infection the crystals were best visualized under the phase microscope. Some late stage nuclei were filled with large crystals. Most nuclei in stage 3 were composed of a well defined central mass of varying sized granules or crystals, encircled by a very clear zone, and a third zone containing large crystals or homogeneous amorphous material (Figs. 9 and 10). The late stage infected cells became markedly rounded, and the nuclear cytoplasmic boundary was often indistinct.

The results of the fluorescent antibody studies of cells in the late stages of infection were somewhat variable. Technical difficulties were caused by the rounding and clumping of affected cells. The thick cell clumps showed diffuse, seemingly non-specific staining; accurate intracellular localization of antigen in such clumps was impossible. Another problem was created by the tendency of affected cells to wash off the glass during the staining procedure. The addition of horse serum (10 per cent by volume) to the maintenance fluid aided in the preservation of cell sheets and partially overcame the cell clumping. Rounding of individual infected cells still occurred, however, and made observation of the cytoplasm very difficult.

Cultures examined 48 hours after infection consistently revealed cells containing large blocky accumulations of viral antigen around the central mass which itself showed varying amounts of specific staining, generally less than that in the surrounding area (see Figs. 15 and 16). Though the outlines of the nucleus were not visible, the blocky masses appeared to correspond to the crystals of stage 3, which are predominantly intranuclear in location. Brightly fluorescent granules were present in the cytoplasm of some of the affected cells as well. In most of the experiments the cultures harvested at 72 hours showed the same cell types and antigen distribution as at 48 hours. One 72 hour experiment differed in that a marked increase in cytoplasmic staining—as well as staining of the nuclear membrane—was evident. The cytoplasmic staining was largely diffuse in nature; very few masses resembling crystals were visible in the fluorescent preparations although they were prominent in companion cultures viewed under the phase and light microscopes.

Although the fluorescent antibody technique demonstrated the presence of specific type 4 adenovirus antigen in the granules and crystalline masses in the nuclei of infected HeLa cells, it could not make plain whether such accumulations of antigens were infectious. In an effort to define the relationship between the development of characteristic microscopic changes and the production of infectious adenovirus, experiments were performed to correlate the two processes.



TEXT-FIG. 1. Multiplication of type 4 adenovirus in HeLa cells and the percentage of HeLa cells showing characteristic cytological changes after type 4 adenovirus infection. Tissue culture tubes, with and without coverslips, were each infected with $10^{1.5}$ TCD_{50} of type 4 virus. For the multiplication curve at each time period, the cells and culture fluid from 4 tubes were harvested and pooled and infectivity titrations performed. The cells on the coverslips were stained with hematoxylin and eosin, or by the fluorescein-labelled antibody technique and the percentage of affected cells determined by counting 1000 or more cells.

In these experiments a large group of tissue culture tubes, with and without coverslips, were infected simultaneously with $10^{1.5}$ to $10^{3.0}$ TCD_{50} of type 4 adenovirus. At selected time intervals after infection coverslips were prepared for microscopic examination by staining with hematoxylin and eosin or with fluorescent antibody. At the same time intervals, companion tissue culture tubes without coverslips were harvested, and after all samples in the experiment had been collected, the titer of infectious virus in each sample was measured. In the preparations stained with hematoxylin and eosin and fluorescent antibody, the percentage of cells showing changes characteristic of type 4 adenovirus infection was determined. At least 1000 cells were counted in each set of fluorescent antibody preparations at each time interval. In the hematoxylin and eosin–stained cultures, because of greater ease and accuracy in counting, a total of 3000 or more cells was counted at each interval.

The results of one correlative experiment, which is representative of several performed, are shown in Text-fig. 1. An increase in infectious virus was first detected 18 hours after inoculation and the incremental period of viral propagation lasted from 30 to 36 hours after infection. Well defined cytological changes and the appearance of specific viral antigen within the nuclei were first detected in a very small percentage of cells (<0.01 per cent) at 16 hours after inoculation. In the preparations stained with hematoxylin and eosin the highest percentage of affected cells (17 per cent) was found at 72 hours after inoculation, but the greatest increase in affected cells occurred between 24 and 38 hours after infection.

The percentage of cells found to reveal characteristic changes was consistently lower in the fluorescent antibody preparations than in the hematoxylin and eosin–stained cultures. In all the experiments performed the counts obtained on the hematoxylin and eosin–stained cells were much more consistent and probably more reliable; the discrepancy between them and the counts obtained on fluorescent antibody–stained cultures appeared to result from the washing off of infected cells during the vigorous rinsing required in the fluorescent staining procedure. Dislodgement of the infected cells presented the greatest difficulty in the late stages, when the cytoplasm of infected cells became rounded and lost much of its contact with the coverslip surface. The small percentage of cells found to contain virus antigen at 72 hours in the one experiment presented above, illustrates the difficulty. In some experiments there was little or no decrease in the apparent percentage of infected cells in the late stage fluorescent antibody–stained cultures.

These experiments demonstrate that there was close parallelism between the time of the appearance of infected cells and the time newly synthesized infectious virus was first detected, and that the period of maximal increase in cytological changes coincided approximately with the incremental phase of viral multiplication. Close comparison and quantitative interpretation of these results are not warranted because of technical differences in the methods used in the experiments and because of the difficulty in accurately determining the percentage of involved cells. The magnitude of the difference in virus

titer in the early and late stages of infection is so great, however, that, even allowing for gross errors in the estimation of the percentage of cells infected and differential loss of late-stage infected cells from the glass, it is evident that the amount of infectious virus per infected cell was much larger in the late stages than in the early. It is not possible to ascertain whether stage 1 cells were productive of small numbers of infectious viral particles or whether the presence of newly produced infectious virus found in the early intervals after infection may be explained by the occurrence of a very few older stages.

No correlation between cytoplasmic fluorescence and high titers of infectious virus was found. The large amount of cytoplasmic staining found in the 72-hour cultures in one experiment was not accompanied by much rise in virus titer (Text-fig. 1). A hundredfold increase in titer, however, was observed between 48 and 72 hours in another experiment in which no appreciable increase in cytoplasmic staining occurred.

DISCUSSION

The correlated immunofluorescent, light, and phase-contrast microscopic studies of type 4 adenovirus-infected HeLa cells have demonstrated that the cells which contain specific viral antigen are the same cells which manifest characteristic morphologic changes. The distribution of the fluorescent staining—and therefore of the viral antigen—corresponds closely with that of certain virus-induced intranuclear structures, such as the regularly aligned granules of the early stages and the crystal-like masses of later forms. Electron-microscopic investigations have revealed that these same structures are composed of crystalline arrays of virus-like particles (3, 20), and cytochemical studies have shown that they contain desoxyribonucleic acid (4–6). Furthermore the experiments reported above showed close temporal correlation between the production of infectious virus and the development of cytological and immunohistochemical changes. There is thus an abundance of evidence which links the intranuclear alterations with adenovirus synthesis.

Results of cell fractionation studies reported in the preceding paper demonstrated that the majority of infectious type 4 adenovirus was isolated from the "cytoplasmic" fraction, an observation which raised some question about the relationship between the intranuclear alterations and viral synthesis. The fluorescent antibody findings described above made untenable the hypothesis that the intranuclear alterations were products of deranged metabolism not directly associated with virus development. It is also unlikely that the changes merely mark the site of previous virus-host cell interaction, for the characteristic alterations develop concurrently with infectious virus. Furthermore, the late crystal-like masses retain their affinity for specific antibody and for basophilic dye, in marked contrast to the late stage inclusion bodies of herpes

simplex infection, which become eosinophilic and lose their complement of virus antigen (21). The possibility remains that much of the antigenic material present in infected nuclei consists of incomplete, non-infectious virus. There is a striking discrepancy between the large amounts of antigenic material demonstrated by fluorescence microscopy and the vast numbers of virus-like particles observed by electronmicroscopy on one hand and the small numbers of infectious units recoverable from each infected cell on the other. It is conceivable that the only infectious viral particles produced are small numbers that undergo maturation after reaching the cytoplasm, although the data presented in this paper do not allow such a conclusion. On the contrary there was close correlation between the nuclear changes and the production of infectious virus, and no consistent shift in the localization of viral antigen from the nucleus in early stages to the cytoplasm in the later forms was observed. There was, however, concentration of viral antigen at the periphery of the nucleus as the crystals amassed in that region, and if the numbers of infectious particles present in the cytoplasm were very small, the methods used in this study might not be sufficiently sensitive to detect them.

It should also be pointed out that there may be other explanations for the discrepancy between the large number of particles observed microscopically and the small number of infectious units recovered. Possibilities which can not be excluded on the basis of available data include aggregation of virus so that one infectious unit represents many infectious particles, incomplete separation of infectious virus from cell components, inactivation of infectious virus coincidental with its release from the cell, and the inadequacy of the techniques employed to determine accurately the number of infectious particles. The data of the cellular fractionation studies (7) suggest that the first possibility, namely aggregation, is the most likely explanation for the discrepancy described.

SUMMARY

HeLa cell cultures infected with adenovirus type 4 were studied by light and phase-contrast microscopy and by the fluorescent antibody technique for visualization of intracellular antigen. The findings were correlated with the growth curve of infectious virus, determined from companion cultures. The results indicated that those cells undergoing characteristic structural changes observable by light microscopy were those which contain viral antigen. The distribution of the majority of the antigen within the infected cells corresponded to that of the regularly aligned granules and crystal-like masses seen in the nuclei of cells in stained and in unfixed cultures. The production of infectious virus was closely correlated with the development of the characteristic nuclear changes.

BIBLIOGRAPHY

1. Kjellen, L., Lagermalm, G., Svedmyr, A. M., and Thorssen, K. G., Crystalline-like patterns in the nuclei of cells infected with an animal virus, *Nature*, 1955, **175**, 505.
2. Harford, C. G., Hamlin, A., Parker, E., and van Ravenswaay, T., Electron microscopy of HeLa cells infected with adenoviruses, *J. Exp. Med.*, 1956, **104**, 443.
3. Morgan, C., Howe, C., Rose, H. M., and Moore, D. H., Structure and development of viruses observed in the electron microscope. IV. Viruses of the RI-APC group, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 351.
4. Barski, G., Caractère spécifique de la lésion cellulaire causée *in vitro* par les virus du groupe A.P.C. et sa valeur diagnostique, *Ann. Inst. Pasteur*, 1956, **91**, 614.
5. Bloch, D. P., Morgan, C., Godman, G. C., Howe, C., and Rose, H. M., A correlated histochemical and electron microscopic study of the intranuclear crystalline aggregates of adenovirus (RI-APC virus) in HeLa cells, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 1.
6. Boyer, G. S., Leuchtenberger, C., and Ginsberg, H. S., Cytological and cytochemical studies of HeLa cells infected with adenoviruses, *J. Exp. Med.*, 1957, **105**, 195.
7. Denny, F. W., Jr., and Ginsberg, H. S., Intracellular localization of type 4 adenovirus. I. Cellular fractionation studies, *J. Exp. Med.*, 1959, **109**, 69.
8. Crouse, H. V., Coriell, L. L., Blank, H., and Scott, T. F. McN., Cytochemical studies on the intranuclear inclusion of herpes simplex, *J. Immunol.*, 1950, **65**, 119.
9. Scott, T. F. McN., Burgoon, C. F., Coriell, L. L., and Blank, H., The growth curve of the virus of herpes simplex in rabbit corneal cells grown in tissue culture with parallel observations on the development of the intranuclear inclusion body, *J. Immunol.*, 1953, **71**, 385.
10. Coons, A. H., and Kaplan, M. H., Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody, *J. Exp. Med.*, 1950, **91**, 1.
11. Hilleman, M. R., and Werner, J. H., Recovery of new agent from patients with acute respiratory illness, *Proc. Soc. Exp. Biol. and Med.*, 1954, **85**, 183.
12. Ginsberg, H. S., Badger, G. F., Dingle, J. H., Jordan, W. S., Jr., and Katz, S., Etiologic relationship of the RI-67 agent to "Acute respiratory disease (ARD)" *J. Clin. Inv.*, 1955, **34**, 820.
13. Scherer, W. F., Syverton J. T., and Gey, G. O., Studies on the propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix, *J. Exp. Med.*, 1953, **97**, 695.
14. Ginsberg, H. S., Gold, E., and Jordan, W. S., Jr., Tryptose phosphate broth as a supplementary factor for maintenance of HeLa cell tissue cultures, *Proc. Soc. Exp. Biol. and Med.*, 1955, **89**, 66.
15. Pereira, H. G., and Kelly, B., Dose-response curves of toxic and infective actions of adenovirus in HeLa cell cultures, *J. Gen. Microbiol.*, 1957, **17**, 517.
16. Boyer, G. S., unpublished data.

17. Weller, T. H., and Coons, A. H., Fluorescent antibody studies with agents of varicella and herpes zoster propagated *in vitro*, *Proc. Soc. Exp. Biol. and Med.*, 1954, **86**, 789.
18. Ginsberg, H. S., Characteristics of the new respiratory viruses (adenoviruses). I. Qualitative and quantitative aspects of the neutralization reaction, *J. Immunol.*, 1956, **77**, 271.
19. Leuchtenberger, C., and Boyer, G., The occurrence of intranuclear crystals in living HeLa cells infected with adenoviruses, *J. Biophysic and Biochem. Cytol.*, 1957, **3**, 323.
20. Low, B. W., and Pinnock, P. R., The packing structure of crystalline RI-APC virus, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 483.
21. Lebrun, J., Cellular localization of herpes simplex virus by means of fluorescent antibody, *Virology*, 1956, **2**, 496.

EXPLANATION OF PLATES

Photomicrographs of HeLa cells illustrating the sequential cellular changes produced by adenovirus type 4. Figs. 1 to 4 are of cells stained with hematoxylin and eosin; Figs. 5 to 10 show fresh, unfixed cells as they appear by phase-contrast microscopy, and Figs. 11 to 16 are of cells stained for specific viral antigen by the fluorescent antibody technique. Photomicrographs of type 3 adenovirus-infected cells, which closely resemble those of cells infected with type 4, were presented in a previous publication (6). Figs. 1 to 4 are given here to allow comparison with the phase-contrast and fluorescence photomicrographs.

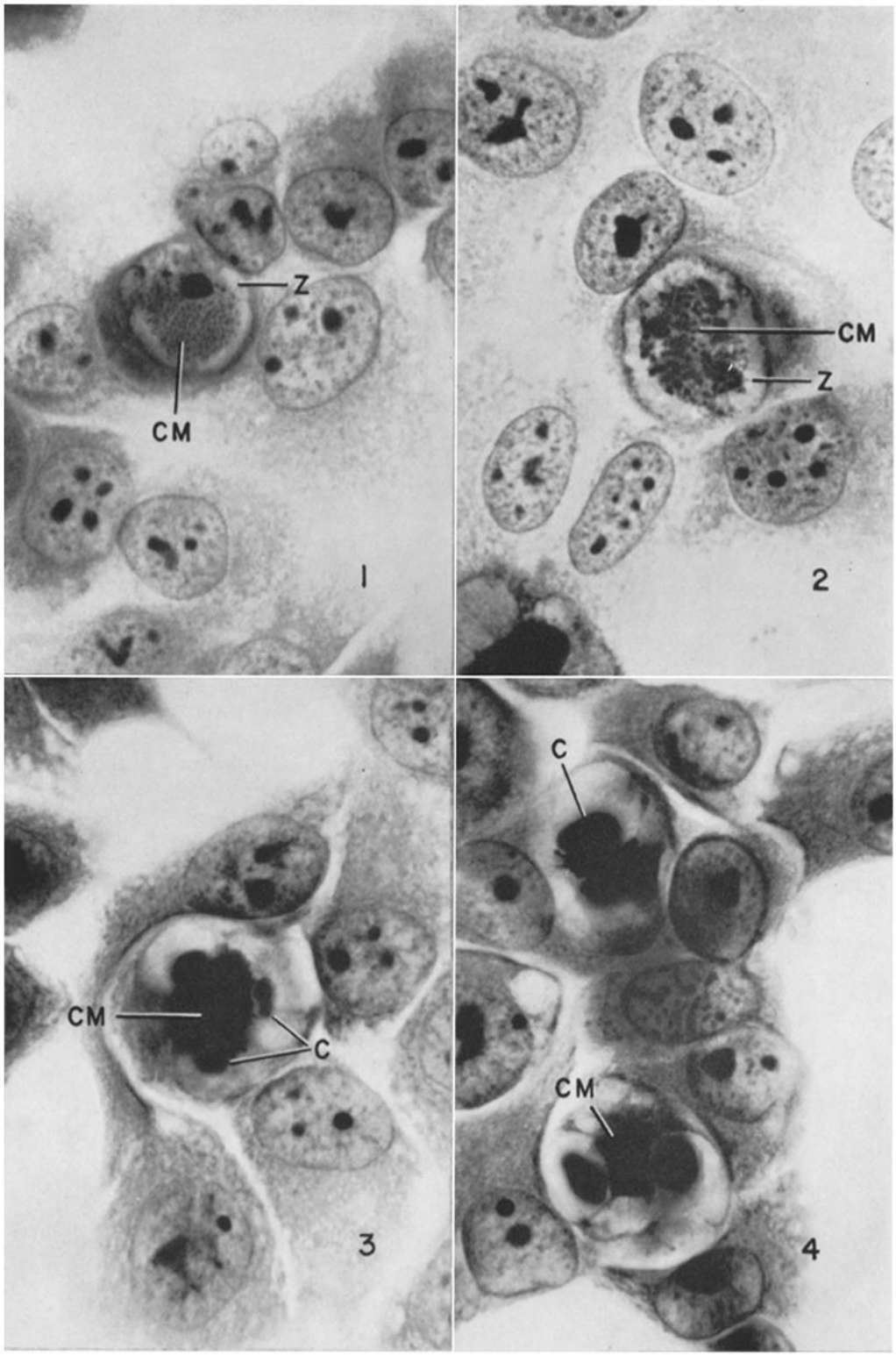
PLATE 10

FIG. 1. HeLa cells 20 hours after infection with adenovirus type 4. The nucleus of the center cell is in stage 1 of the cytologic sequence. The chromatin network has been replaced by a central mass (*CM*) of uniform granules, and a clear zone (*Z*) is evident beneath the nuclear membrane. The cytoplasm of the infected cell appears intensely stained; it has retracted, giving the cell a rounded outline. Compare with surrounding unaffected cells. Hematoxylin and eosin. × 1000.

FIG. 2. HeLa cells 30 hours after infection, showing a nucleus in stage 2. The central mass (*CM*) consists of small discrete masses of varying size, larger than the granules in the stage 1 nucleus in Fig. 1. The peripheral zone (*Z*) is now wider. Hematoxylin and eosin. × 1000.

FIG. 3. HeLa cells 48 hours after infection, including a rosette form characteristic of stage 3. The central mass (*CM*) is surrounded by a wide peripheral nuclear zone of bubble-like compartments containing crystal-like masses (*C*). Hematoxylin and eosin. × 1000.

FIG. 4. Cells 72 hours after infection, including two stage 3 rosette forms with intensely stained central masses (*CM*) and large basophilic crystalline inclusions (*C*). Note variation in crystal size; compare Figs. 3 and 4. Hematoxylin and eosin. × 1000.



(Boyer *et al.*: Type 4 adenovirus. II)

PLATE 11

FIG. 5. Fresh, unfixed HeLa cells 20 hours after infection, including cell in stage 1 of cytologic change. Nucleus of infected cell is filled with regularly spaced granules (*G*). Compare with Fig. 1. Phase-contrast. × 800.

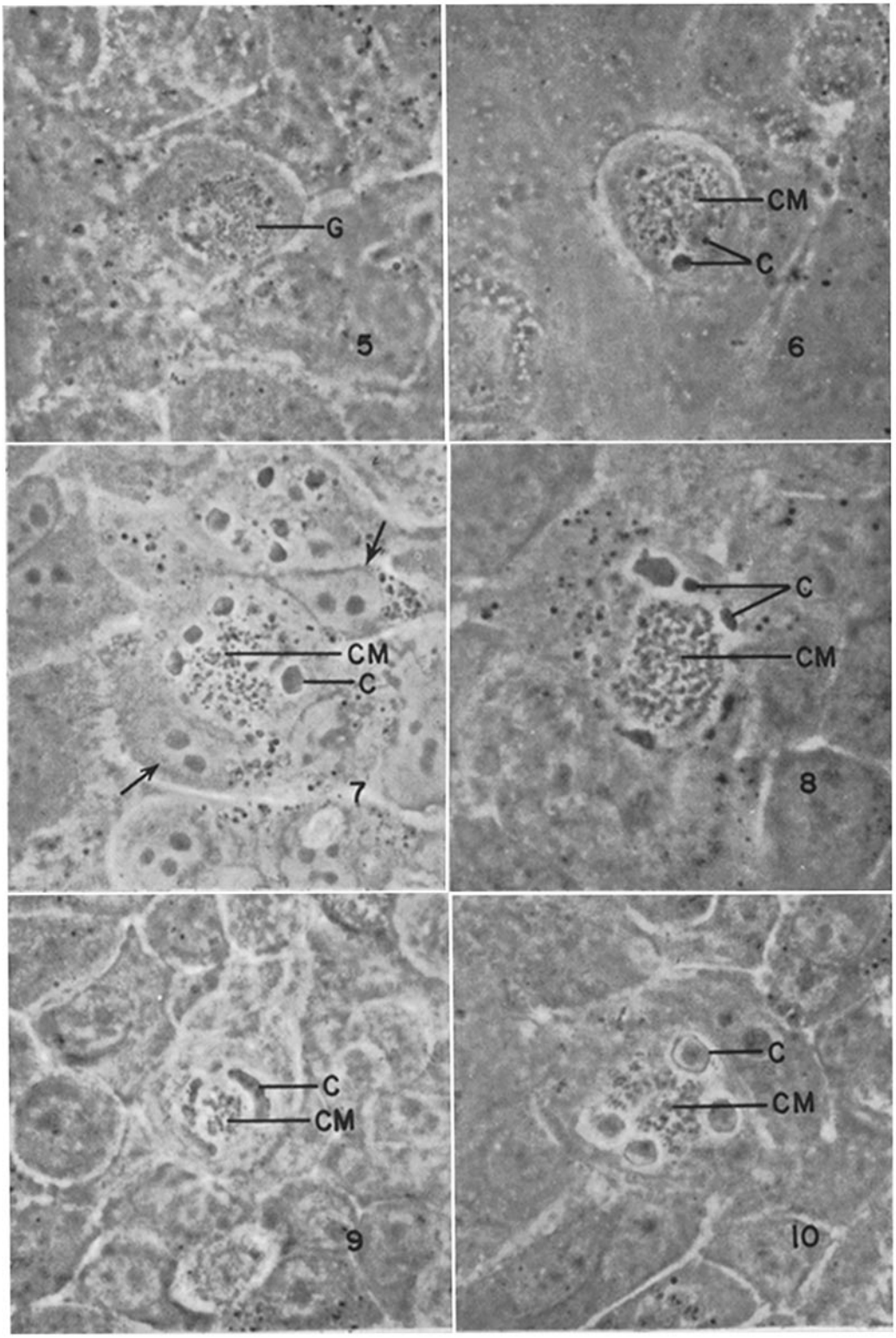
FIG. 6. HeLa cell in transition between stage 1 and stage 2, 24 hours after infection. Homogeneous crystal-like masses (*C*) appear at the edges of the nucleus and among the granules of the central mass (*CM*). Compare with Fig. 2. Phase-contrast. × 800.

FIG. 7. HeLa cell nucleus in stage 2, 30 hours after infection, with granular central mass (*CM*) surrounded by crystals (*C*) concentrated in the nuclear periphery. Compare with Fig. 13. Arrows indicate unaffected nuclei containing rounded homogeneous nucleoli. Phase-contrast. × 800.

FIG. 8. HeLa cell nucleus late in stage 2, 36 hours after infection. The granular central mass (*CM*) is separated from the encircling crystal-like masses (*C*) by a clear zone. Compare with Fig. 14. Phase-contrast. × 800.

FIG. 9. Stage 3 nucleus, 48 hours after infection, with small central mass (*CM*) and elongated, poorly-defined crystals (*C*). Compare with Figs. 3 and 15. Phase-contrast. × 800.

FIG. 10. Stage 3 cell, 48 hours after infection, with large, well defined crystals (*C*) in the peripheral nuclear zone around the central mass (*CM*). Bubble-like compartments of this rosette form are not well illustrated in the photograph, but were apparent in a different plane of focus. Compare with Figs. 4 and 16. Phase-contrast. × 800.



(Boyer *et al.*: Type 4 adenovirus. II)

PLATE 12

FIG. 11. HeLa cell culture 18 hours after infection, showing cell in stage 1. The nucleus is filled with brightly fluorescent granules, indicating the site of virus antigen. Compare with hematoxylin and eosin–stained stage 1 nucleus, Fig. 1, and unfixed nucleus in Fig. 5. Indirect fluorescent antibody stain. × 1300.

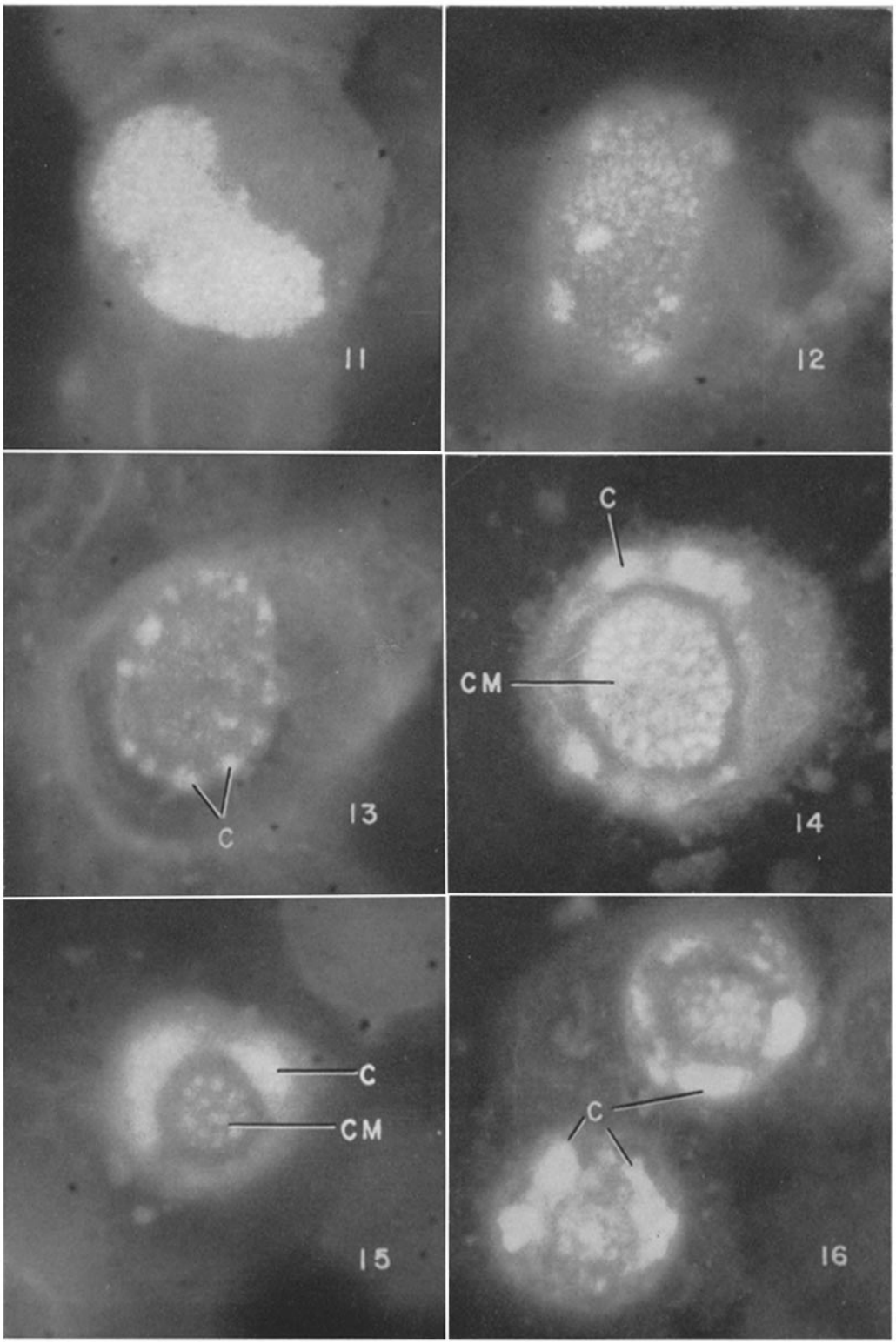
FIG. 12. Twenty-four hours after infection, showing a nucleus in transition between Stages 1 and 2. In addition to regularly spaced granules, larger accumulations of antigenic material (crystal-like in form) are dispersed through the nucleus. Compare with Figs. 2 and 6. × 1300.

FIG. 13. Thirty hours after infection. Stage 2 nucleus shows clustering of brightly stained antigen-containing crystal-like masses (*C*) at the nuclear periphery. Compare with stage 2 nuclei in Figs. 2 and 7. × 1300.

FIG. 14. Thirty-eight hours after infection. The brightly stained central mass (*CM*) is separated from the surrounding crystal-like masses (*C*) by a clear unstained zone. Compare with Fig. 8. × 1300.

FIG. 15. Forty-eight hours after infection, showing a stage 3 nucleus with a central mass (*CM*) and large, poorly defined crystals (*C*). A clear zone encircles the central mass. Compare with Figs. 3 and 9. × 1300.

FIG. 16. Forty-eight hours after infection. Two stage 3 cells with large crystal-like masses (C) with heavy concentrations of antigen, and less brightly stained central zones. Compare with Figs. 4 and 10. × 1300.



(Boyer *et al.*: Type 4 adenovirus. II)