CYTOLOGICAL AND CYTOCHEMICAL STUDIES OF HeLa CELLS INFECTED WITH ADENOVIRUSES*

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Plates 20 to 23

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The isolation of a new group of viruses from the respiratory tract of man (1, 2) has served as a stimulus for numerous investigations in recent years. Fourteen immunologically distinct virus types (3), now termed "adenoviruses" (4), have been discovered and incorporated into a single group on the basis of a common complement-fixing antigen (5). Several members of this group have been found to act as etiologic agents in human illness (2, 6–9).

It was the purpose of the experiments described in this report to characterize by cytological and cytochemical means the changes induced in host cells by four types of adenovirus, all of which are known to be cytopathogenic agents (2, 6). These studies were performed in conjunction with other investigations concerning the relationship of adenoviruses to their host cells in tissue culture (10, 11). Light microscopy offered two advantages as an approach for studying the effects of the viruses upon the cells. First, it permitted the examination of large numbers of cells, necessary in establishing the steps in a sequence of viral induced changes. Second, it enabled the study to be made of cytochemical as well as morphological alterations in virus-infected cells.

Several investigators have reported the results of electron microscopic studies of HeLa cells infected with adenoviruses (12–15). All have described the occurrence of regular rows of virus-like particles, which may be arranged in crystalline patterns, within the nuclei of infected cells. Correlation of these electron microscopic findings with the present data, obtained by means of

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light microscopy, contributes to the understanding of both viral development and host cell reaction to infection.

This report describes the alterations in HeLa cells infected with types 1 to 4 adenoviruses. All of these agents were found to produce consistent and characteristic cytological and cytochemical changes in the nuclei; the alterations caused by types 1 and 2 viruses were significantly different, however, from those produced by types 3 and 4 viruses.

Materials and Methods

Viruses.—The viruses employed were the prototype strains of adenovirus-types 1, 2, and 3 ["adenoid degeneration" agents or "adenoidal-pharyngeal-conjunctival" agents (6)] isolated by Rowe, Huebner, Gilmore, Parrott, and Ward (1) and the type 4 virus (RI-67 agent) isolated by Hilleman and Werner (2). All the viruses were propagated by serial passage in HeLa cells according to methods previously described (16).

Tissue Culture.—Cells of the HeLa strain, originally derived by Gey from an epidermoid carcinoma of the cervix, were propagated in tissue culture by a modification of the methods described by Scherer, Syverton, and Gey (16, 17). Stock cell cultures were carried in 32 ounce duraglas bottles; such cultures were started with 30 ml. of a suspension containing 5×10^4 cells per ml. All cell suspensions were made by mechanical means without the use of trypsin. For cell cultivation, a nutritive medium composed of 40 per cent human serum and 60 per cent Hanks's balanced salt solution was employed.

For cytological and cytochemical studies cells were grown on strips of coverslips (6 x 22 mm. in size) which had been cleaned in alcohol and ether, placed in 16 x 150 mm. screw cap tubes, and autoclaved prior to use. 0.5 ml. of a suspension of HeLa cells containing 10^5 cells/ml was added to each tube. The tubes were then slanted in stationary racks and incubated at 36°C. for 24 hours to allow the cells to adhere to and spread on the glass. Prior to viral inoculation each culture was washed twice with 2 ml. of Hanks's balanced salt solution; 0.8 ml. of maintenance mixture was added. The maintenance mixture employed consisted of Scherer's amino acid-vitamin solution (17), 67.5 per cent; chicken serum, 7.5 per cent; and tryptose-phosphate broth, 25 per cent (18).

Virus Infection of HeLa Cells.—Serial 1:3.2 ($10^{-0.5}$) dilutions of the stock virus were made in Hanks's balanced salt solution. Each culture was inoculated with 0.1 ml. of the chosen virus dilution. Undiluted, $10^{-1.0}$, $10^{-1.5}$, $10^{-2.0}$ and higher dilutions were tested, but a 10^{-2} dilution was most frequently employed. Infectivity titrations were done with serial 1:3.2 ($10^{-0.5}$) dilutions of virus suspensions as previously described (16).

Method of Following the Cellular Changes.—At different time intervals after infection, varying from 6 hours to 7 days, the coverslip preparations were removed from the culture tubes for fixation and staining. The most thoroughly investigated intervals were 14, 16, 18, 20, 22, 24, 30, 36, 42, 48, 60, 72, and 96 hours after infection.

In the course of the present investigation 40 experiments were performed and a total of 617 coverslip cultures examined. These included 188 control cultures (of which 143 were stained with hematoxylin and eosin and 46 by the Feulgen technique) and 429 infected cultures (352 hematoxylin and eosin and 77 Feulgen preparations). Approximately equal numbers of cultures were infected with each of the four viruses studied. In experiments comparing the effects of the different agents, cultures of the same origin and age were used. Carefully controlled, uniform techniques were used throughout the preparation of cells for cytological study.

Fixation and Staining.—Ninety-five per cent ethyl alcohol was usually employed for fixation of cells to be stained with hematoxylin and eosin; 3 to 5 minute periods of fixation sufficed.

Cells stained with hematoxylin and eosin after fixation with 10 per cent neutral formalin, with Bouin's fluid, and with Carnoy's fixative (3 parts absolute ethyl alcohol and 1 part glacial acetic acid) were also examined.

In order to visualize desoxyribonucleic acid, the Feulgen reaction was carried out under standardized and controlled conditions (19).

Carnoy's fixative was generally employed for the Feulgen preparations; 95 per cent ethyl alcohol was used for fixation in some experiments. A dilute alcoholic solution of fast green (approximately 0.001 per cent) was used when a counterstain was desired.

The specificity of the Feulgen reaction for DNA in the preparations under study was determined by the following control studies: (*a*) the absence of positive staining in both infected and uninfected cultures pretreated with desoxyribonuclease, and (*b*) the absence of positive staining in cultures not subjected to acid hydrolysis before exposure to Schiff's reagent.

Controls.—Parallel uninfected cultures were made with each set of infected coverslip cultures; these controls were derived from the same stock bottles and were of the same age as the infected cells. Uninfected cultures were handled in exactly the same manner as the infected cells, except for actual viral inoculation, throughout the experiment.

Mitotic Counts.—Mitotic rates were calculated from counts made on 10 sets of coverslip preparations, each set consisting of one or two infected cultures and an uninfected control culture of the same age. Cultures were examined early and late after infection, and a total of 38,000 cells were counted. These counts were made independently by three observers.

Microspectrophotometric Techniques.—For the quantitative determinations of DNA in individual nuclei of the uninfected and infected HeLa cells, Feulgen microspectrophotometry was used. The validity of Feulgen microspectrophotometry for DNA determinations has been well established (19, 20).

EXPERIMENTAL OBSERVATIONS

Before describing in detail the characteristic morphological and cytochemical alterations occurring in infected cells, two aspects of the cell-virus relationship should be mentioned.

The first concerns the effects of varying concentrations of virus on HeLa cells. Highly concentrated viral suspensions, although desirable in order to initiate synchronous infection, produced rounding, clumping, and detachment of cells from the glass very early after inoculation. The capacity to produce such effects was retained by concentrated inocula which had been heated for 5 minutes at 56°C. (21). This heat treatment, however, did abolish (*a*) the ability to synthesize new virus, as determined by infectivity titrations and (*b*) the ability to induce the sequence of characteristic nuclear alterations described below. Since these effects peculiar to concentrated suspensions were undesirable for the observation of cytological changes and were apparently distinct from viral synthetic processes, more dilute inocula ($10^{-1.5}$ and 10^{-2}) were employed in most of the experiments. Variation of the concentration of virus added to the cell cultures did not affect the characteristic nuclear changes *per se*, but did influence the length of the interval after infection at which the first alterations could be detected and the rapidity with which the changes progressed.

The second aspect of the host-virus relationship to be discussed deals with

the proportion of cells affected with time. In infected cultures showing the earliest stages in the sequence of cytological alterations (beginning at 14 hours after inoculation) only a small proportion of the cells appeared to be affected. The proportion of involved cells increased markedly with time. The time at which the maximum per cent of cells were involved varied with the concentration of and to some extent with the type of virus used. In none of the cultures studied did all the cells appear to be affected. Even in the very late stages of the infection (5 to 7 days) there were always a number of cells that resembled the controls in all respects; some were undergoing mitosis.

Appearance of HeLa Cell Control Cultures.—When grown in maintenance mixture (which lacks human serum) HeLa cells were polygonal and arranged in closely packed sheets. Cytologically they had the typical appearance of tumor cells, exhibiting disparity in cellular and nuclear size and shape, large multiple nucleoli, and many mitotic figures, often of abnormal configuration (see Figs. 1 and 2). In hematoxylin and eosin-stained preparations the nucleoli were strongly basophilic, whereas in Feulgen preparations they appeared as clear areas surrounded by a Feulgen-positive rim of nucleolus-associated chromatin. Occasional pyknotic and karyorrhectic cells were observed; such cells increased in number with the age of the culture.

Although the cytoplasm was usually homogeneous and acidophilic, it was sometimes vacuolated, had alternating areas of increased density and rarefaction, and peripheral protrusions. Infrequently a mass of brightly eosinophilic debris was seen within the cytoplasm.

Despite the lack of uniformity of the HeLa cells in the control cultures studied, the microscopic appearance was extremely consistent in different cultures of varying ages. Cells maintained for 7 days in the maintenance mixture showed no important differences from cells incubated only 16 hours in the same fluid.

Appearance of HeLa Cell Cultures Infected with Types 1 and 2 Adenoviruses

The cytological alterations observed after infection with the types 1 and 2 viruses were so similar that they will be discussed together. One of the most conspicuous features in HeLa cell cultures infected with these agents was the reduction in the rate of mitosis. As early as 18 hours after infection a decrease in the mitotic rate of approximately 40 to 50 per cent could be detected. Between 30 and 33 hours after infection the mitotic rate had decreased further to approximately 20 to 30 per cent of the values obtained in uninfected cultures of the same age.

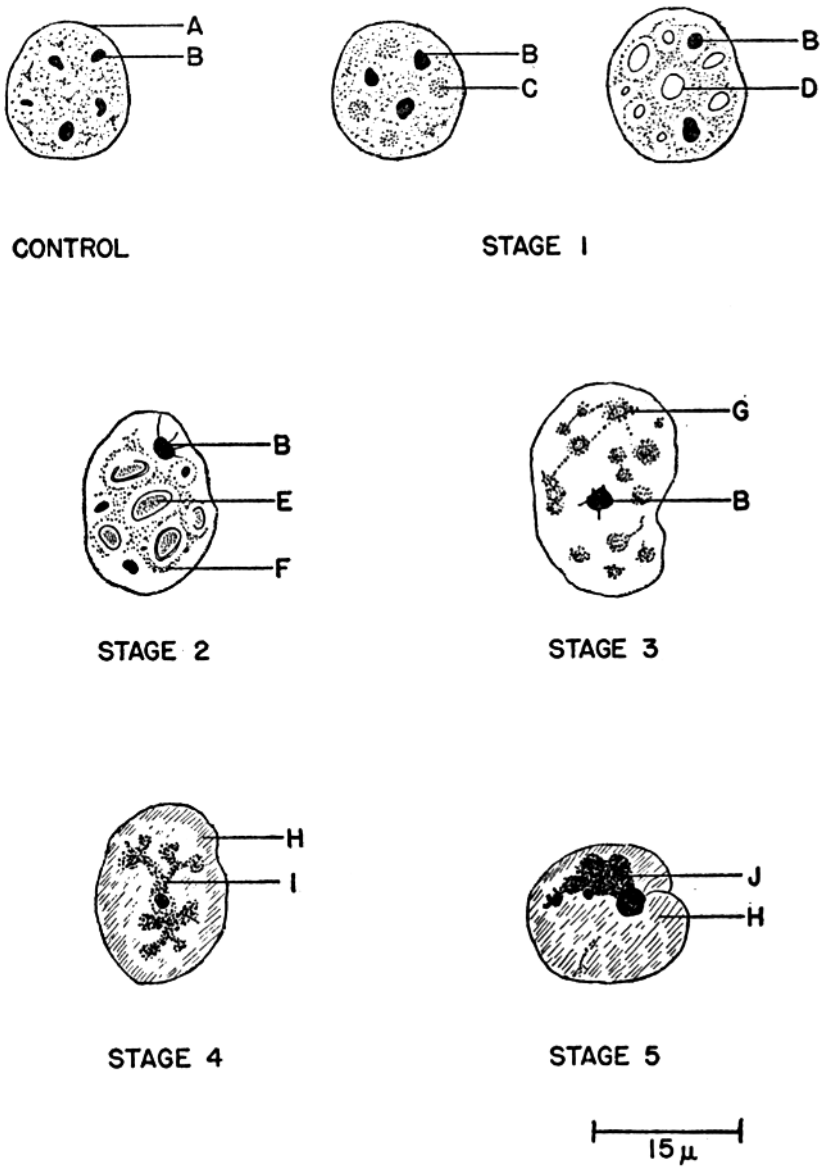
The description of the alterations observed in the individual cells will be concerned predominantly with the nuclei, in which the most striking and consistent changes occurred. For the cytological studies both hematoxylin

and eosin and Feulgen preparations were used. The latter were examined in order to investigate the participation of desoxyribonucleic acid in the sequential alterations observed in companion cultures stained with hematoxylin and eosin. Since the onset of the cytologic alterations was not synchronous, a variety of altered cell types were present in most of the infected cultures studied. Cultures fixed at the extremes of the "cycle" of changes (*e.g.*, at 14 hours and 72 hours after infection) showed far less variation in the cytological picture; the first and last steps in the sequence of cytological changes were therefore established more easily than the intervening ones. With the observation of many cells at frequent intervals after infection, however, the remaining major steps in the sequence of cytologic changes became apparent. For convenience of description the sequence of changes progressing with time has been divided into five stages (see Text-fig. 1).

Stage I.—Fourteen to 20 hours after infection with $10^{2.5}$ to $10^{3.0}$ infectious doses of either type 1 or 2 adenovirus two kinds of unusual structures could be observed within a small proportion of the nuclei: (*a*) aggregates of eosinophilic granules surrounded by a clear zone, and (*b*) round or oval eosinophilic bodies, each with a sharply outlined basophilic rim. (See Text-fig. 1, Stage I, and Fig. 3) These structures could be easily distinguished from nucleoli, present in the same cells. The eosinophilic bodies varied in size, were multiple in number (generally from 3 to 10 per nucleus), and often contained fine eosinophilic granular elements. In parallel Feulgen preparations these bodies did not appear to be Feulgen-positive, although the rims of some of the inclusions seemed to contain DNA. The appearance of eosinophilic inclusions was usually accompanied by redistribution of the chromatin and increase in nuclear size.

Stage II.—Approximately 19 to 27 hours after infection a higher proportion of the cells contained the nuclear structures described in Stage I. In addition, in numerous other nuclei the inclusions themselves exhibited a variety of alterations. The number of eosinophilic inclusions occasionally increased to 20 or more. They were sometimes arranged in bead-like fashion or in clusters; they often seemed to have coalesced, forming delicate honeycomb-like structures or granular masses. The eosinophilic granulation within many of the inclusions was replaced by a basophilic core; a rarefied zone appeared between the core and the rim (see Text-fig. 1, Stage II and Fig. 4). In companion slides clusters of Feulgen-positive granules could be seen which were similar in size and distribution to these inclusions (see Fig. 5). The regular arrangement of granules in masses formed by coalesced inclusions was particularly marked in Feulgen preparations. The rearrangement of chromatin and increase in nuclear size were more pronounced than in Stage I.

Stage III.—This stage, which predominated between 29 and 36 hours after infection, was characterized by the appearance of many discrete basophilic



TEXT-FIG. 1. Diagrammatic representation of HeLa cell nuclei infected with adenovirus type 1 or 2 (hematoxylin and eosin stain). (*A*) nuclear membrane, (*B*) nucleolus, (*C*) aggregate of eosinophilic granules, (*D*) eosinophilic body, (*E*) basophilic core of eosinophilic inclusion, (*F*) rearranged chromatin, (*G*) basophilic granular cluster, (*H*) glassy background, (*I*) merging clusters, and (*J*) dense basophilic mass.

granular clusters spaced throughout the nuclei (see Text-fig. 1, Stage III, and Fig. 6). These clusters, which seemed to be derived from the more basophilic inclusions of Stage II and the rearranged chromatin, were Feulgen-positive (Fig. 7). Eosinophilic inclusions could still be seen in some nuclei, though often in a collapsed or broken state. During this third stage the most striking increase in nuclear size, associated with an apparent increase in quantity of Feulgen-positive material, was observed (see Text-fig. 1, Stage III, Figs. 6 and 7). The increase in size occurred in the majority of cells in the culture.

Stage IV.—From 36 to 48 hours after infection many cells showed a decrease in nuclear size, beginning fusion of the basophilic granular clusters, and darker staining of the nuclear background (see Text-fig. 1, Stage IV). The background stained with both hematoxylin and eosin, producing a homogeneous, glassy effect. This background had a light, but definitely *positive* Feulgen reaction (see Fig. 8).

Stage V.—During the advanced stages of infection the cells clumped together and heaped up, and many became detached from the coverslip. These changes, first evident during Stage IV, progressed in Stage V (from 48 hours after infection to the completion of observations at 4 to 7 days) until only a small percentage of the cells remained on the glass. The nuclei of the affected cells left usually contained one or more dense, basophilic, and Feulgen-positive masses lying within the glassy background (see Text-fig. 1, Stage V, and Figs. 9 and 10). In many affected nuclei, the nucleoli, which persisted throughout the course of the nuclear changes could still be seen. A small proportion of the nuclei showed degenerative changes, such as pyknosis, which may be seen in cells dying from causes other than viral infection.

Since the cytoplasmic alterations in cells infected with type 1 or 2 virus did not differ markedly from those in cells infected with type 3 or 4 adenovirus, the changes of this sort are described below in conjunction with the cytological effects of the latter agents.

Appearance of HeLa Cell Cultures Infected With Adenovirus Types 3 and 4

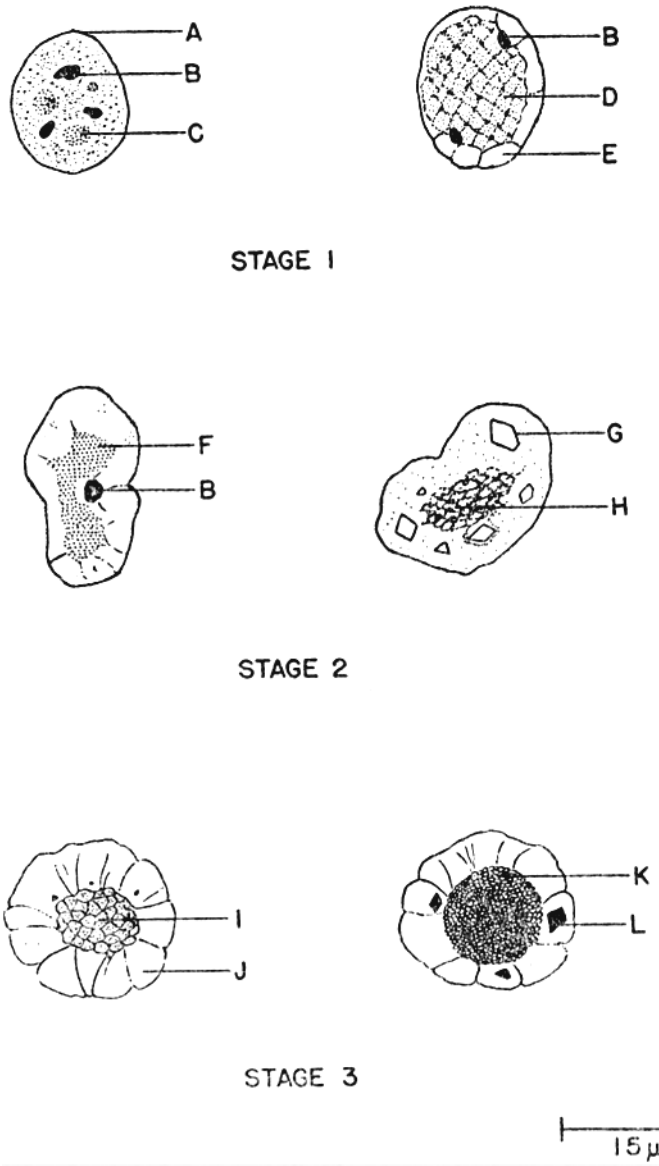
Cells infected with type 3 or 4 adenovirus deviated markedly in appearance from both control cells and cells infected with either type 1 or 2 adenovirus. Some differences were observed between cells infected with type 3 and those infected with type 4 virus. For example, despite efforts to match inocula in terms of infectious doses the progression of changes was more rapid, and the loss of polygonal outline more pronounced after infection with the type 4 virus. Nevertheless, the main features shown by cells infected with these two agents were sufficiently similar to permit simultaneous description.

Stage I.—In hematoxylin and eosin preparations the first characteristic cytological alterations could be detected 14 hours after infection with $10^{2.0}$ to $10^{3.0}$

infectious doses of either virus. A small number of nuclei contained one or more finely stippled eosinophilic masses. Other nuclei possessed a single large granular central mass, usually separated from the nuclear membrane by a rarefied zone (see Text-fig. 2, Stage I, and Figs. 11 and 12). The central mass had definite structure, different from that of the interphase chromatin of uninfected cells. Within the central mass were Feulgen-positive granules, which often formed a delicate network or a densely packed array (see Fig. 14).

Stage II.—Approximately 30 hours after infection a variety of additional cellular changes could be noted. The nuclei, sometimes greatly increased in size, often showed extreme distortion of shape as well (see Figs. 12, 14, and 16). In some nuclei the central zone resembled a coarse, beaded network or regularly granular mass (see Text-fig. 2, Stage II, and Fig. 12); in others, a fine lattice work or strikingly regular honeycomb (see Text-fig. 2, Stages II and III). All of these structures were Feulgen-positive (see Fig. 14). Nucleoli, easily visualized in Stage I, were no longer apparent in many of the nuclei in Stage II (see Text-fig. 2, Stage II). The peripheral nuclear zone, now frequently increased in width, varied in appearance. In some cells it seemed almost devoid of stain or contained only scattered granules; in others it had a homogeneous eosinophilic hue. The peripheral nuclear zones of other cells contained striking angular inclusions with sharp edges (see Text-fig. 2, Stages II and III, and Figs. 13 and 15). These inclusions frequently seemed crystalline in outline (they will be referred to as "crystals"). These "crystals," which were sometimes surrounded by a web of basophilic matter (see Fig. 13), varied in size, shape, and staining properties. They were best visualized after fixation with alcohol; in cells fixed with Carnoy's or with Bouin's fluid the "crystals" seemed far fewer in number, and those that were present were often so distorted that their identification was difficult. It appeared that the acid present in the fixatives had a deleterious effect on the crystal-like structures. That the "crystals" were not artifacts produced by fixation with alcohol has been clearly shown by study of living cells; crystal-like structures were well visualized in infected cells under the phase-contrast microscope and under the interference microscope. Crystals were not seen in accompanying control cells (22). In cultures stained with hematoxylin and eosin the "crystals" varied from brightly eosinophilic to basophilic. Eosinophilic "crystals" were more prominent in cultures infected with adenovirus type 3 than in those infected with type 4. A corresponding array of reactions, from negative to intensely positive, could be observed in the "crystals" of cells in companion Feulgen preparations (Fig. 17). Treatment with desoxyribonuclease abolished the positive Feulgen reaction of the "crystals," but did not destroy the "crystals" nor significantly alter their structure.

Stage III.—Approximately 48 hours after infection with type 4 virus and 96 hours after inoculation with type 3, nuclei showing further cellular alterations



TEXT-FIG. 2. Diagrammatic representation of HeLa cell nuclei infected with adenovirus type 3 or 4 (hematoxylin and eosin stain). (*A*) nuclear membrane, (*B*) nucleolus, (*C*) eosinophilic mass, (*D*) delicate network type central mass, (*E*) rarefied zone, (*F*) regularly granular central mass, (*G*) eosinophilic crystal, (*H*) coarse network type central mass, (*I*) honeycomb type central mass, (*J*) compartment of flower form, (*K*) dense central mass, and (*L*) basophilic crystal.

became increasingly numerous. Stage III was characterized by the presence of a large, intensely stained basophilic and Feulgen-positive mass which, unlike pyknotic chromatin, showed evidence of definite structure (see Text-fig. 2, Stage III, and Fig. 18). The central mass of some nuclei was seen to consist of an agglomeration of small crystals; in others the mass had a honeycomb-like appearance. Frequently, however, the mass was too dense and too darkly stained to allow observation of detailed structure. In some cells there were ovoid or wedge-shaped compartments radiating out from the central mass, producing a flower-like nuclear form (see Text-fig. 2, Stage III, and Figs. 18 and 19). The peripheral nuclear zone in other cells was not subdivided into compartments, but was stippled with fine, regular eosinophilic granules. Sometimes it had a glassy appearance similar to that of cells infected with type 1 or 2 virus. Crystalline inclusions, usually basophilic and Feulgen-positive, were frequently seen around the central mass. In some Stage II and III cells the nuclear membrane could not be discerned or did not appear intact (see Figs. 13 and 19).

Changes observed in the cytoplasm of infected cells were by no means as distinctive as the nuclear alterations. Furthermore, as mentioned above, few differences could be found between the cytoplasm of cells infected with type 1 or 2 viruses and the cytoplasm of those infected with type 3 or 4 adenovirus. Cultures fixed in Bouin's fluid were particularly suitable for the study of the cytoplasm. Although the changes described below were consistently observed, and occurred with much greater frequency in infected cells, similar ones were seen occasionally in uninoculated cultures.

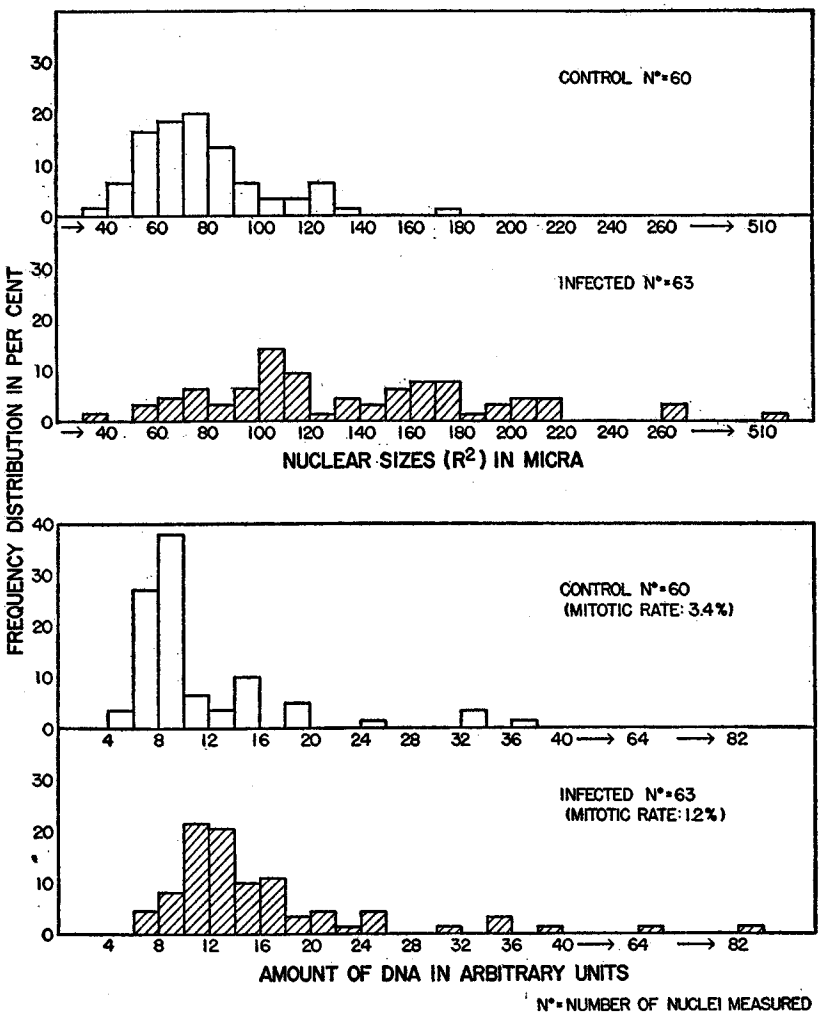
Among the cytoplasmic alterations observed in cells with characteristic early nuclear changes were: (*a*) increased heterogeneity, (*b*) vacuolization, (*c*) blurring of the cellular margins, and (*d*) protrusions from the cell surface. In the later stages a densely granular eosinophilic zone was often noted surrounding the nuclei of cells infected with adenovirus type 1 or 2. In cells infected with type 3 or 4 virus, especially after alcohol fixation, a dense basophilic rim of cytoplasm was often seen encircling the nucleus. With the increase in nuclear size there was no concomitant increase in cytoplasmic mass. On the contrary, the latter appeared to decrease. In the final stages of infection the cytoplasm showed degenerative changes and disintegration; frequently only shreds remained (see Fig. 9).

Cytoplasmic inclusions containing DNA have not been observed in Feulgen preparations, except in late stages of infection with either type 3 or 4 virus when the "crystals" sometimes appeared to be extranuclear.

Quantitative Determinations of Desoxyribonucleic acid by Feulgen Microspectrophotometry

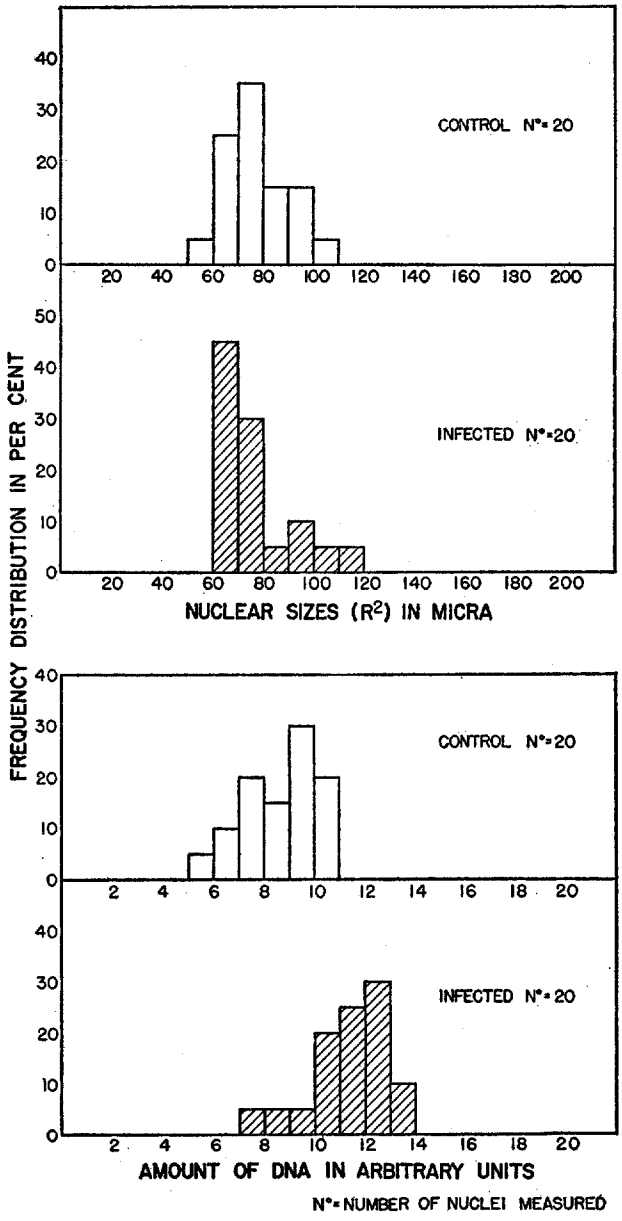
Since on cytological examination, the nuclei of infected HeLa cells appeared to show an increase not only in size, but also in Feulgen-positive material,

quantitative determinations of the DNA in individual nuclei were made by Feulgen microspectrophotometry. The two examples presented in Text-figs.



TEXT-FIG. 3. Nuclear sizes of and the amounts of DNA (Feulgen microspectrophotometry) in large interphase nuclei from control HeLa cell cultures and from HeLa cell cultures 30 hours after infection with $10^{2.5}$ to $10^{3.0}$ infectious doses of adenovirus type 1.

3 and 4 are the initial studies in an extensive microspectrophotometric investigation now in progress which is concerned with a correlation between the sequential cytological alterations and the DNA changes in cells infected with adenoviruses.



TEXT-FIG. 4. Nuclear sizes of and the amounts of DNA (Feulgen microspectrophotometry) in interphase nuclei from control HeLa cell cultures and HeLa cell cultures 72 hours after infection with $10^{2.0}$ to $10^{8.0}$ infectious doses of adenovirus type 3.

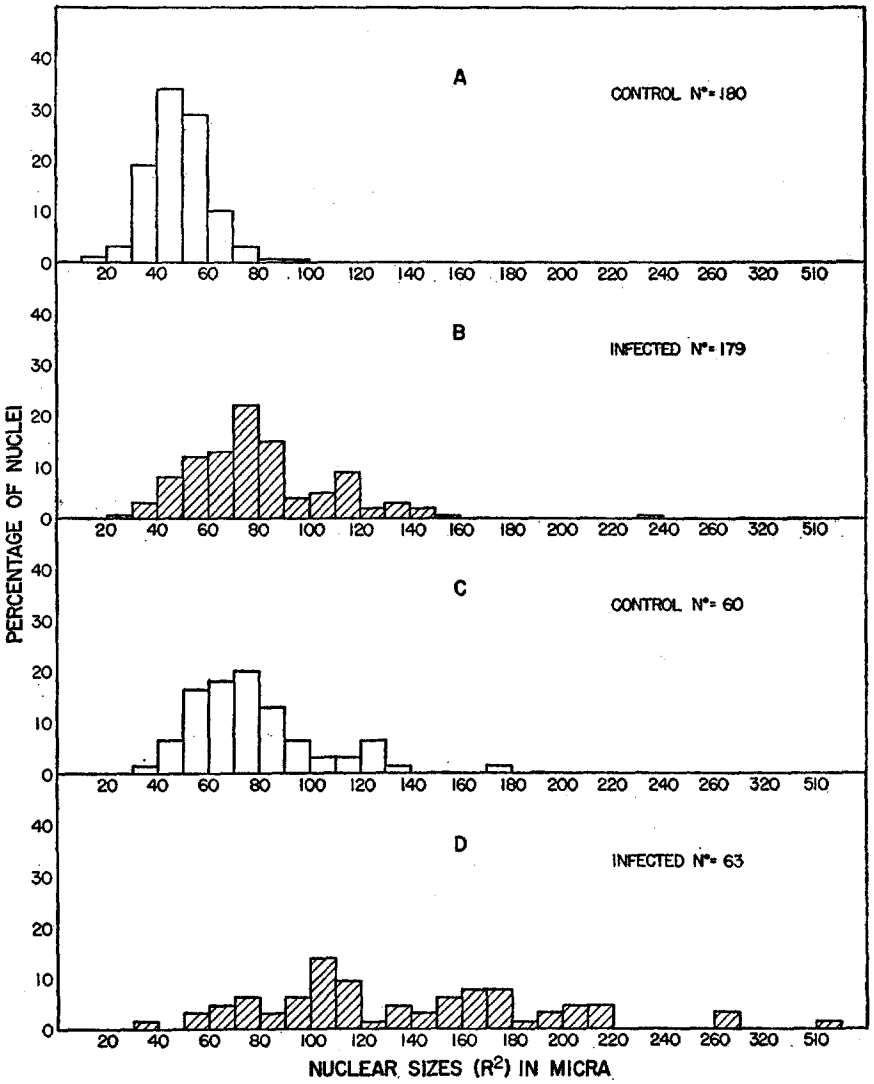
Random selection of nuclei for DNA measurements was not feasible in the material under study since some nuclei, particularly infected ones, were unsuitable for microspectrophotometric determinations because of marked irregularity of shape and/or of DNA distribution (19). In the first microspectrophotometric study (Text-fig. 3) the largest interphase nuclei available were selected from both control cultures and cultures infected with adenovirus type 1. Such a selection seemed indicated to assure that the build-up of DNA in interphase nuclei during mitosis and the large amounts of DNA occurring in rare giant control nuclei were not overlooked. It should be emphasized again that one of the characteristic features of cells infected with adenovirus type 1 was nuclear enlargement, most marked during Stage III. The upper two graphs in Text-fig. 5 illustrate this phenomenon of increased size of infected nuclei. These graphs depict the relative nuclear sizes of cells from uninfected control cultures and from cultures 30 hours after infection with adenovirus type 1.

In obtaining data for Graphs A and B, 360 nuclei from 8 separate Feulgen preparations were measured, and the average diameter and radius of each nucleus calculated. The square of the radius was taken as an index of the nuclear area. In the control preparations all interphase nuclei encountered were measured consecutively, beginning with a field selected at random; in the infected preparations all apparently infected nuclei (including early, mid-, and late stages) were measured in the same manner. The data obtained from measuring cells in four different uninfected cultures were in close agreement (means of the 4 samples: 48.23, 48.64, 48.27, 45.41). Greater variation was found in the infected cells, but consistently from culture to culture the mean area of the infected nuclei was larger than that of the uninfected nuclei.

Graphs C and D show the nuclear areas of the cells in which DNA determinations were made illustrating the large size of the nuclei selected from both control and infected cultures.

In Text-fig. 3 the nuclear sizes and the individual DNA values are graphed for large nuclei from cultures 30 hours after infection with adenovirus type 1 and for large nuclei from control cultures of the same age. The DNA values of the majority of the control cells measured show a peak between 6 and 10 units, that is, an approximate doubling from the tetraploid to the octaploid value. This range of DNA values is to be expected on the basis of the well known findings that during mitosis doubling of DNA occurs at interphase (23, 24). In a sample of nuclei selected for large size a high proportion of cells preparing for division would be anticipated. The DNA values of the infected cells did not reveal a similar peak, but showed a wider scatter and were generally higher. Some infected nuclei contained up to ten times the average DNA value and two and one-half the highest DNA value obtained in the control cells.

Change in the DNA content was also found in HeLa cells infected with adenovirus type 3 (Text-fig. 4). Here, in both infected and control cultures interphase nuclei of approximately the same size were selected for DNA determinations.



TEXT-FIG. 5. Comparison of nuclear sizes of uninfected HeLa cell nuclei and nuclei of HeLa cells 30 hours after infection with $10^{3.5}$ to $10^{3.9}$ infectious doses of adenovirus type 1. Graphs *A* and *B* refer to consecutively counted nuclei. Graphs *C* and *D* refer to the nuclei selected for microspectrophotometric measurements.

It is evident that despite the similarity in nuclear sizes, the DNA values in the majority of the infected cells measured were definitely higher than those of the control cells.

That infected interphase nuclei contain the same or even greater amounts of DNA than control nuclei seems surprising when the reduction of mitotic

rate in infected cultures is considered. For example, the mitotic rate of the infected cell culture for which the DNA values are graphed in Text-fig. 3 was reduced to approximately 30 per cent that of the uninfected control. Consequently, the number of interphase nuclei doubling their DNA content because of mitotic activity should have been smaller in the infected culture than in the control; that is, a shift toward lower DNA values might be expected in the infected cells. Since there was no shift toward lower DNA values, but rather

TABLE I

Summary of Alterations in HeLa Cells Infected with Types 1 to 4 Adenovirus

Feature	Types 1 and 2	Types 3 and 4
Inhibition of mitosis	Present	Present, less marked under conditions employed
Time at which characteristic nuclear changes first appear	14–16 hrs. ($10^{2.5}$–$10^{3.0}$ ID_{50})	14 hrs. ($10^{2.0}$–$10^{3.0}$ ID_{50})
Well demarcated early intranuclear eosinophilic inclusions	Consistently seen	Rarely present
Central nuclear mass	Seen only when inclusion bodies coalesce	Constant feature
Discrete Feulgen + clusters spaced through nuclei	Conspicuous feature consistently seen	Rarely seen
Nuclear enlargement	Pronounced, involved majority of cells	Present, but less pronounced and more variable
Wrinkling of nuclear membrane, distortion of nuclear shape	Present	Present
Crystalline inclusions (Feulgen-negative to positive)	Very rarely seen	Prominent feature
Glassy, Feulgen + nuclear background	Consistently seen	Present in some cells
Flower-like nuclear configuration	Infrequent	Frequent, characteristic feature
Increase in DNA	Present	Present
Apparent decrease in cytoplasmic mass	Seen	Seen

toward higher ones in the infected cells measured, the results assume special significance in relation to synthesis of DNA by viral multiplication. Whether the higher DNA quantities observed were due to multiplying virus DNA or due to an unusual and irregular synthesis of nuclear DNA induced by the presence of the virus cannot be decided with certainty from the data available.

DISCUSSION

In order to facilitate discussion certain salient aspects of cells infected with adenovirus types 1 to 4 are summarized in Table I. All the cytological features

described, including those listed in Table I, occurred with a high degree of reproducibility in many repeated experiments. Nuclear alterations characteristic of cells in infected cultures were not found in any of the control cultures examined. The consistency of response renders more significant the deviations in the cytological appearance of cultures infected with adenovirus type 1 or 2 from those of cultures infected with type 3 or 4. This variation in response of the same cell line to types 1 and 2 viruses on the one hand, and types 3 and 4 on the other, is in accord with other data suggesting that these four agents may be classified into 2 subgroups (10). The initial cycle of multiplication is similar for types 1 and 2 viruses, and the quantitative relationships of these two agents with their respective type-specific neutralizing antibodies are identical. Furthermore, these characteristics of types 1 and 2 viruses are distinctly different from those of types 3 and 4, which are in turn similar to each other (10, 11).

In the interpretation of the cytological alterations the question arises as to whether the changes observed within the nuclei of cells in infected cultures are merely manifestations of non-specific cell damage and abnormal metabolic activities or whether they actually reflect the development of virus particles. Several lines of evidence point toward the tenability of the latter alternative: (*a*) the nature of the cytological and cytochemical findings presented in this study (see Table I), (*b*) the correlation of these findings with those obtained by electron microscopy (12–15) and (*c*) the correlation of the observations presented in this report with studies of viral reproduction in tissue culture (10, 21). Each of these lines of evidence will be discussed in detail below.

The occurrence of well defined inclusion bodies and regular patterns of granules, the accumulation of aggregates and the appearance of crystalline masses within the nuclei are all consistent with the presence of intranuclear virus. That these structures do not actually exist but merely represent fixation artifacts is most unlikely. The major features of infected cells, with the exception of the "crystals" observed after type 3 or 4 infection, were little affected by variation in the fixative employed. Furthermore, phase microscopic examination of unfixed infected cells has confirmed the presence of the inclusions characteristic of adenovirus type 1 or 2 infection and the central masses, flower forms, and crystal-like inclusions typical of type 3 or 4 infection.

The chemical nature of the characteristic nuclear structures provides additional support for the presence of intranuclear virus. With the exception of the early stages of the inclusions and granular aggregates and some of the "crystals," DNA can be demonstrated in all the characteristic structures by means of the Feulgen reaction. The increased amounts of intranuclear DNA found in some of the infected nuclei suggest the occurrence of viral synthesis, particularly when compared with evidence from other instances of intranuclear virus multiplication (19, 25).

Further corroboration that the changes observed reflect viral propagation lies in the similarity of the cytological and cytochemical behavior of cells infected with adenoviruses to that of cells infected with other viruses, such as herpes simplex (26, 27) and the polyhedra virus of the silkworm (28), in which intranuclear viral synthesis is believed to occur. Especially striking parallels are found in the alterations induced in the cells of the silkworm by the polyhedra virus: these alterations include nuclear swelling and the formation of a large, central intranuclear mass surrounded by polyhedral crystals containing DNA (28). (Compare with Text-fig. 2, Stage III, and Fig. 19.) The character of the cytological and cytochemical changes described seems to be typical of and almost specific for virus-infected cells; the appearance of the nuclei of cells engaged in growth, mitosis, or other metabolic activity or of cells which are undergoing necrosis and pyknosis is significantly different. During the course of this study uninfected HeLa cell cultures were subjected to various conditions and agents (*e.g.*, nutritional deficiency and "toxic" doses of heat-inactivated virus) which caused the cells to clump and detach from the glass surfaces on which they grew. Such damaged cells did not reveal nuclear alterations which resembled those characteristic of cultures infected with adenoviruses.

The results of electronmicroscopic studies (12–15) also support the belief that the cytological changes described in this report are manifestations of intranuclear viral synthesis. Electron photomicrographs of HeLa cells infected with adenoviruses reveal many virus-like particles in crystalline arrays within the nuclei. The "crystals" visible under the light microscope in cells infected with adenovirus type 3 or 4 may correspond to the large crystalline aggregates of virus-like particles observed in electron microscopic preparations (14). A recent report of electron microscopic observations of HeLa cells infected with adenoviruses (15) states that crystal-like arrangement of the virus-like particles was uncommon with types 1 and 2, a finding which is also in accord with the light microscopic studies reported here.

Additional indication of the specificity of the cytological changes and their relation to viral synthesis can be derived from correlating the cytological changes with the results of multiplication studies of the four viruses (21). Precise experiments correlating the stages in the sequence of cytological alterations with the multiplication characteristics of the viruses have not been accomplished on identical cultures. Viral multiplication studies were done, however, with the same viruses and cell line, and under conditions very similar to those of the cytological studies. Comparison of the cytological findings with data from the multiplication studies reveals certain facts: (*a*) nuclear changes in a small proportion of the cells could be detected during the latent period of the initial multiplication cycle, and (*b*) the maximal cytological alterations occurred during the period of maximal viral multiplication.

Considering then that the sequential changes observed may reflect actual

viral presence and synthesis, speculations on the significance of the changes in infected cultures can be made:—

1. A marked reduction of the mitotic rate was found in infected cultures. This phenomenon can be explained in several ways. A toxic effect of the virus might be responsible. The explanation might lie in the channeling of host energy and nucleoprotein building blocks into the production of virus rather than into division processes. Indeed chromatin which has already been formed might be broken down and used for viral synthesis.

2. A review of the alterations in cells produced by both "subgroups" of adenoviruses studied reveals a trend from early affinity for acid dye and negative Feulgen reaction toward increased affinity for basic dye and positive Feulgen reaction. This sequence suggests the initial laying down of a basic protein, with the later addition of acidic components, of which DNA appears to constitute an important part. It is not inferred that the early eosinophilic matter is devoid of DNA, for it may well be present in concentrations too low to be detected by the Feulgen reaction under the conditions employed. Unlike the inclusions observed in the nuclei of cells infected with herpes simplex which pass through a Feulgen-positive stage and subsequently become Feulgen-negative (26), the nuclei of HeLa cells infected with adenoviruses do not appear to lose their intense Feulgen-positive nature, even in the latest stages of viral infection examined (5 to 7 days).

The data obtained in this study do not provide any direct information on the mode of viral entry of exit from the cell, nor do they aid in the evaluation of the role of the cytoplasm in viral development. The results of these studies, particularly when correlated with electronmicroscopic findings, do suggest that synthesis of these adenoviruses occurs within the nuclei of infected cells; they do not permit the conclusion that the nuclei contain antigenically complete, infectious virus. It is hoped that investigation of infected cells by means of the fluorescent antibody technique (29) and other cytochemical reactions, now in progress, will provide additional information on the nature of the inclusions and other structures characteristic of adenovirus infection as well as further insight into the development of the adenoviruses.

SUMMARY

The sequential morphological and cytochemical alterations in HeLa cells infected with adenovirus types 1 to 4 are described. Each of the four viruses studied led to consistent and reproducible cytological changes not observed in uninfected control cultures. All four agents produced striking and characteristic changes in the nuclei of infected cells. Alterations in the cytoplasm, though present, were less marked, particularly in the early stages of infection. In cells infected with type 1 or 2 adenovirus, rounded intranuclear inclusions which progressed from eosinophilic and Feulgen-negative to basophilic and Feulgen-

positive, together with a homogeneous glassy, Feulgen-positive nuclear background, were prominent features. Cells infected with type 3 or 4 adenovirus exhibited marked rearrangement of basophilic nuclear material and sharply defined crystal-like inclusions, predominantly intranuclear in location, which also varied from Feulgen-negative to positive. In terms of detailed cytological effects, therefore, the four agents could be divided into two subgroups, *viz.*, types 1 and 2 on the one hand and types 3 and 4 on the other. Measurement of DNA in individual nuclei by means of Feulgen microspectrophotometry revealed the values in infected cells to be increased above the levels of the uninfected controls.

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EXPLANATION OF PLATES

Photographs of HeLa cells infected with adenovirus type 1 (Figs. 1 to 10) and with adenovirus type 3 (Figs. 11 to 19). All hematoxylin and eosin preparations shown were fixed in 95 per cent alcohol; all Feulgen preparations shown were fixed in Carnoy's fixative. None of the Feulgen preparations have been counterstained.

Plate 20

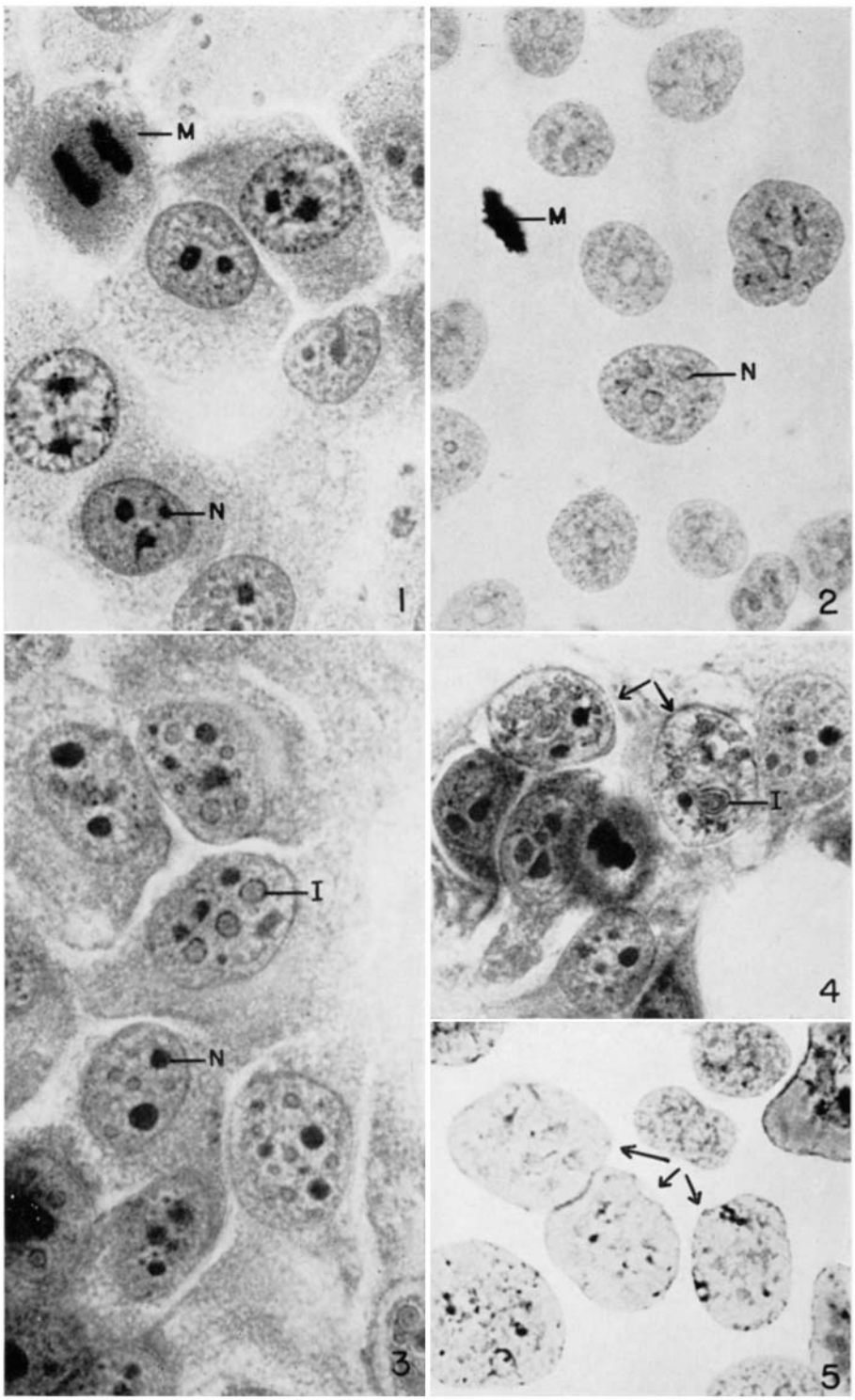
Fig. 1. Uninfected HeLa cells including a mitotic figure (*M*). Note homogeneous cytoplasm. Nucleoli (*N*) are multiple, dark staining. Hematoxylin and eosin. × 800.

Fig. 2. Control HeLa cell nuclei, including one metaphase mitotic figure (*M*). Nucleoli (*N*) outlined by Feulgen-positive rim. Feulgen reaction. × 800.

Fig. 3. HeLa cells 20 hours after infection with adenovirus type 1. Stage I, showing multiple eosinophilic inclusions (*I*) within the nuclei. Rims of inclusions are basophilic. Note dark staining nucleoli (*N*) in same cells. Hematoxylin and eosin. × 900.

Fig. 4. Cells 24 hours after infection with adenovirus type 1. Two cells in Stage II (arrows). Inclusions (*I*) show rim separated from basophilic core by clear zone. Note also increased rearrangement of chromatin, heterogeneity of cytoplasm. Hematoxylin and eosin. × 900.

Fig. 5. Nuclei of cells in Stage II (arrows) 28 hours after infection with type 1 virus. Note scattered Feulgen-positive masses; compare with inclusions in Figs. 3 and 4. Feulgen reaction. × 900.



(Boyer *et al.*: HeLa cells infected with adenoviruses)

PLATE 21

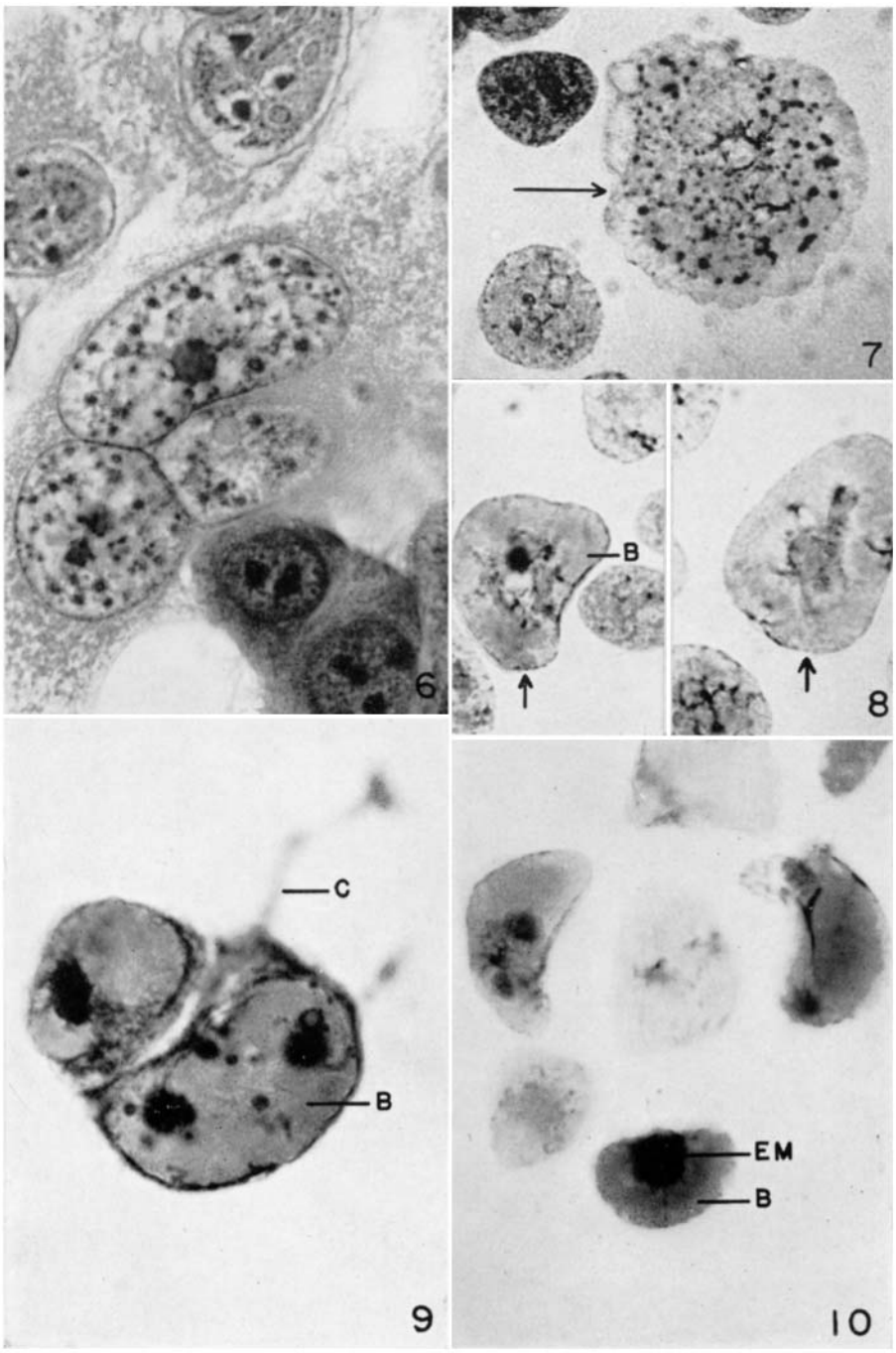
FIG. 6. HeLa cells 32 hours after infection with adenovirus type 1. Note greatly enlarged nuclei in Stage III, showing numerous basophilic granular clusters and enlarged nucleoli. Note contrast in size of normal appearing nuclei in lower right of same figure. Hematoxylin and eosin. × 900.

FIG. 7. Nuclei 35 hours after infection with type 1 virus. Stage III nucleus (arrow) showing enlargement, irregularity of outline, and scattered Feulgen-positive masses. Compare with Fig. 6. Feulgen reaction. × 800.

FIG. 8. Stage IV nuclei (arrows), 42 hours after infection with type 1 virus. Note glassy, Feulgen-positive nuclear background (*B*) and merging granular clusters. Feulgen reaction. × 800.

FIG. 9. Stage V, 72 hours after infection with type 1 virus. Note intensely stained eccentric masses, glassy nuclear background (*B*). Cytoplasm (*C*) scant and disintegrating. Hematoxylin and eosin. × 900.

FIG. 10. Nuclei in Stage V, 72 hours after infection with type 1 virus. Eccentric mass (EM) is intensely Feulgen positive; nuclear background (*B*) also positive. Feulgen reaction. × 750.



(Boyer *et al.*: HeLa cells infected with adenoviruses)

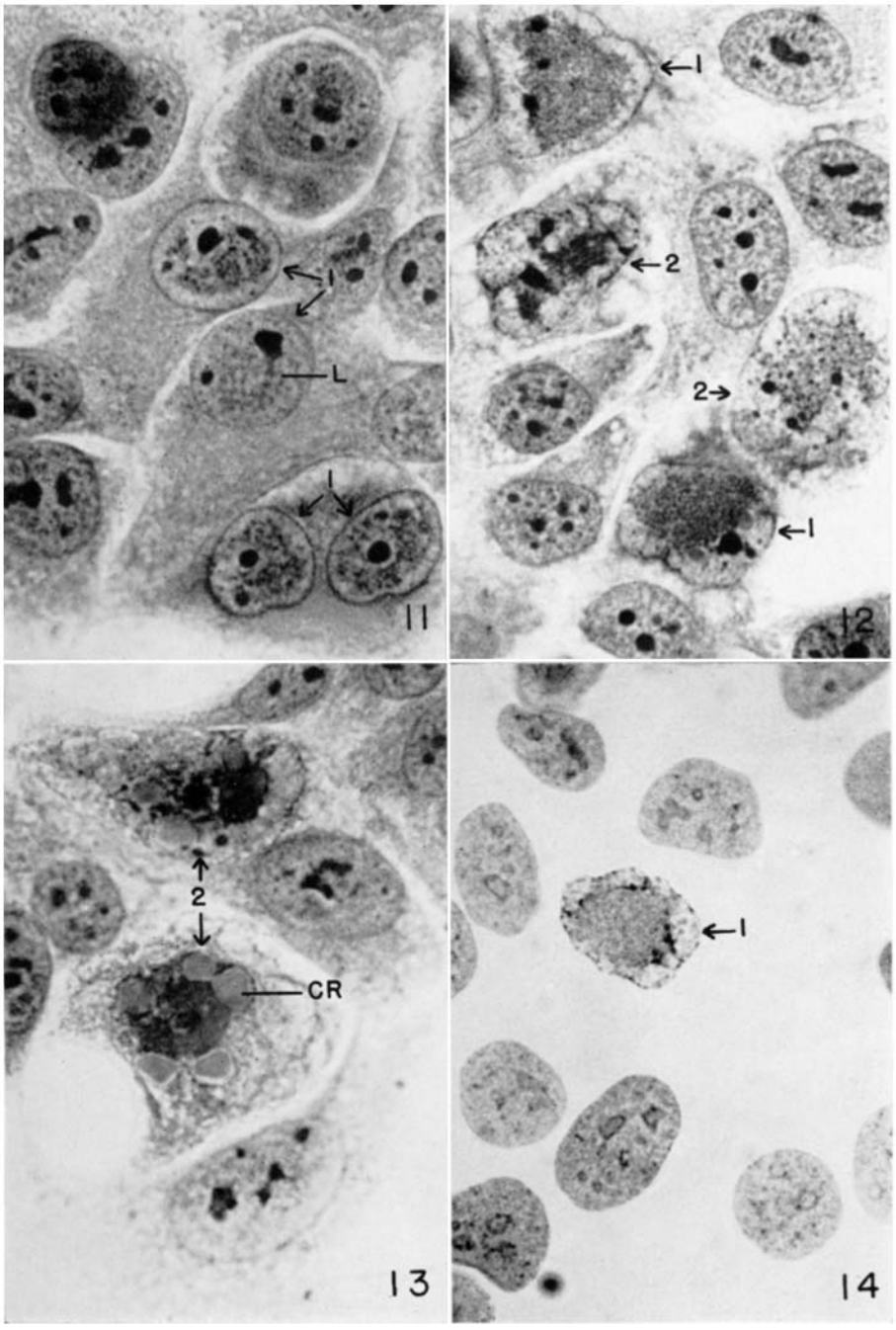
PLATE 22

FIG. 11. HeLa cells 20 hours after infection with adenovirus type 3. Nuclei (arrows) in Stage I (*1*). Note rarefied peripheral zone beneath nuclear membrane. Note difference in appearance of granular intranuclear material from that of normal interphase nuclei in Fig. 1. Note also lattice formation (*L*) in center cell. Hematoxylin and eosin. × 900.

FIG. 12. HeLa cells 36 hours after infection with type 3 virus, showing 2 transitional forms between Stage I and II (*1*) and 2 Stage II nuclei (*2*). Note evenly granular nuclear masses of transition forms, rarefied peripheral nuclear zones, persistence of nucleoli. Affected nuclei are large and irregular in outline. Cytoplasm is vacuolated and heterogeneous. Hematoxylin and eosin. × 900.

FIG. 13. HeLa cells 60 hours after infection with adenovirus type 3. Two Stage II cells (*2*) containing eosinophilic crystalline inclusions (*CR*). Web of basophilic material surrounds several of the crystals. The nuclear boundaries in affected cells cannot be distinguished. Hematoxylin and eosin. × 900.

FIG. 14. Nuclei 30 hours after infection with type 3 adenovirus. Stage I (*1*) nucleus with rarefied peripheral zone and regularly granular Feulgen-positive central mass. Compare with Figs. 11 and 12. Feulgen reaction. × 900.



(Boyer *et al.*: HeLa cells infected with adenoviruses)

PLATE 23

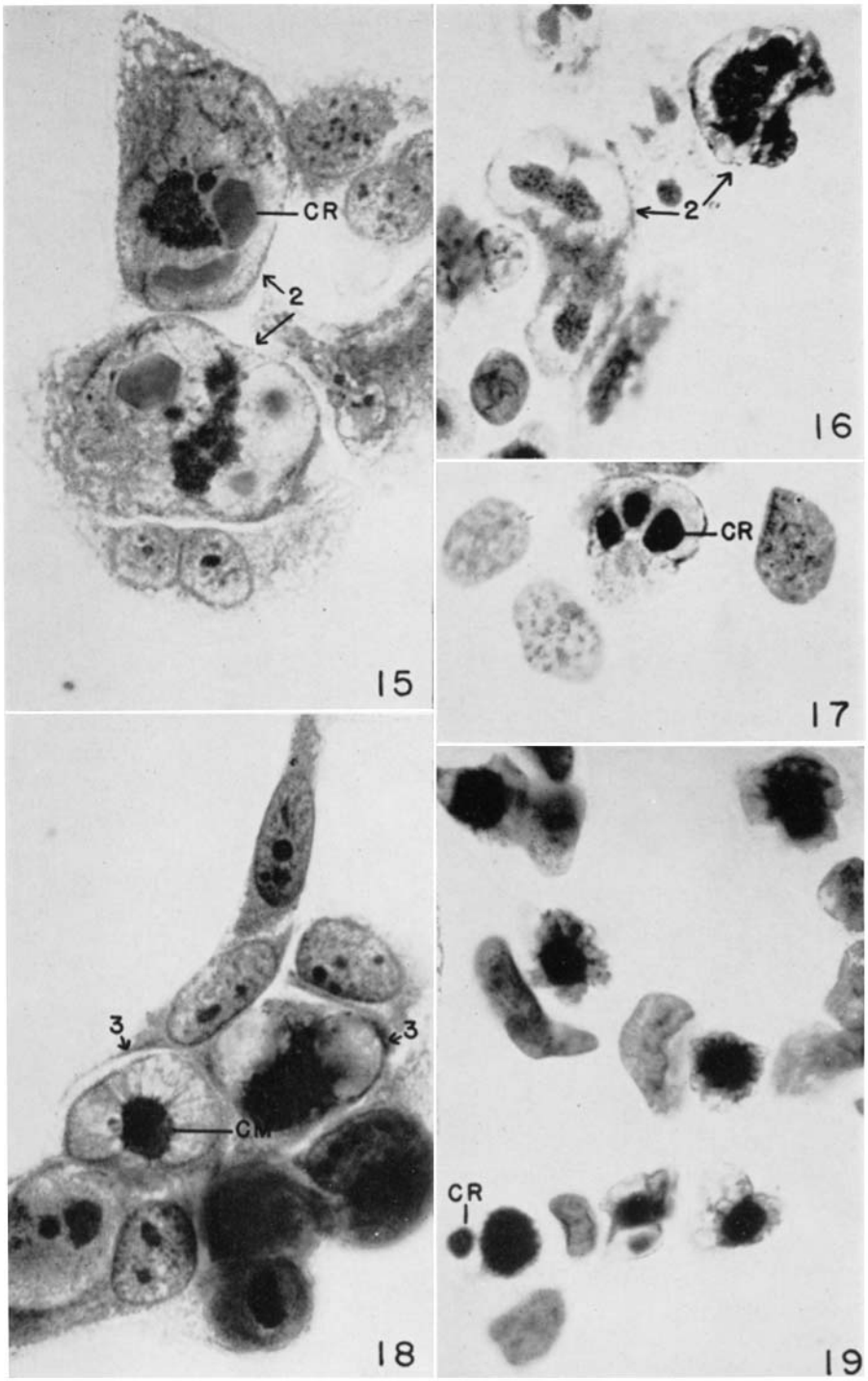
FIG. 15. HeLa cells 71 hours after infection with adenovirus type 3. Late Stage II (*2*) nuclei containing basophilic crystalline inclusions (*CR*). Compare size of Stage II nuclei with that of surrounding apparently normal nuclei. Note dark-staining, heterogeneous cytoplasm. Hematoxylin and eosin. × 800.

FIG. 16. Nuclei 70 hours after infection with adenovirus type 3. Stage II (*2*) forms (arrows) showing intensely positive Feulgen reaction of central masses. Note distortion of nuclear shape. Feulgen reaction. × 800.

FIG. 17. Nuclei 72 hours after infection with adenovirus type 3. Late Stage II form showing 8 intensely Feulgen-positive crystalline inclusions (*CR*). Angles of "crystals" somewhat blunted. Compare with Fig. 15. Feulgen reaction. × 800.

FIG. 18. Cells 78 hours after infection with adenovirus type 3. Arrows indicate typical Stage III (*3*) nuclei. Nucleus at left shows the flower configuration with compartments radiating out from central mass (*CM*). Hematoxylin and eosin. × 900.

FIG. 19. Nuclei 90 hours after infection with adenovirus type 3. Seven Stage III flower forms are visible. Compare intensity of Feulgen reaction with that of apparently unaffected nuclei in the same field. Note Feulgen positive crystal (*CR*). Feulgen reaction. × 500.



(Boyer *et al.*: HeLa cells infected with adenoviruses)