A CORRELATIVE STUDY BY ELECTRON AND LIGHT MICROSCOPY OF THE DEVELOPMENT OF TYPE 5 ADENOVIRUS

II. Light Microscopy*

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The cellular lesions provoked in cell cultures by adenoviruses of the first seven serotypes have hitherto been scrutinized in fixed and stained preparations (1–13) and in infected living cells (6, 9, 10, 12, 14) with the light microscope and its phase and fluorescence adaptations. From such studies it appears that the changes produced in cultured cells by adenoviruses are chiefly dependent on viral type, and that they fall into two general patterns.

In the course of studies on the behavior of adenoviruses in cell cultures of human amnion and of the HeLa and HEp-2 lines it was found that type 5 brings about striking nuclear alterations, differing from those provoked by types 3 and 7, with the formation of both acidophilic and basophilic bodies and large angulated intranuclear crystals (5, 14). Such infected cells have been examined with the electron microscope and with histochemical methods. Morphological interpretation of these nuclear alterations becomes most meaningful when descriptions of the same structures seen with the resolution of the electron microscope can be correlated with those seen in the light microscope, permitting some knowledge of their chemical nature to be derived by application of histochemical methods. In order to gain insight into the cellular response to adenoviral infection and the processes of viral synthesis and release, it is necessary to establish the order in which the changes occur. It has previously been shown (4, 9, 13), and reascertained in these experiments, that inocula of type 5 virus of concentration 10^{-1} to 10^{-2} initiate a single cycle of nearly parasynchronous infection in amnion, HeLa, and HEp-2 cells through a period of about 48 hours, during which all the successive events in the morphologic development of the

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lesion can be traced. In this way Boyer, Leuchtenberger, and Ginsberg (4) studying types 1 to 4 and Nasz and Toth (11) and Boyer, Denny, and Ginsberg (12) studying type 5 have followed a sequence of successive stages as they occur with time in the evolution of the infection. This procedure has also been followed in the present study, with the intention of integrating certain of the electron microscopic and histochemical findings, and the stages in the development of the lesions.

Materials and Methods

1. Cell cultures of the HeLa and of the HEp-2 (FJ) lines were maintained in a modified Hanks-Eagle solution containing inactivated horse serum. For cytological and histochemical studies cells were grown on rectangular coverslip strips (about 6 × 20 mm. in size) affixed to the flat surfaces of Leighton tubes. Each culture was inoculated with 0.1 ml. of standard $10^{-1.5}$ dilution of stock virus. In the time sequence and most of the other experiments the AD-75 strain of type 5 adenovirus was employed and HEp-2 cells were infected; in subsequent experiments strain 196 (Rowe) was also inoculated into both HEp-2 and HeLa lines and the cytopathic effects it produced compared with those of the AD-75 strain.

2. Whole amounts of monolayered cells grown on the coverslips in Leighton tubes were fixed in either absolute methanol or neutral phosphate-buffered formalin. Some were subjected to the freezing-substitution procedure with absolute ethanol as substituent (15).

Pellets of cells obtained from flask cultures by scraping or trypsinization were fixed in neutral formalin, veronal-buffered osmium tetroxide, Carnoy's fluid, or a mixture of absolute ethanol, chloroform, and trichloracetic acid in the proportions 6:3:1; some were frozen-substituted. Pellets were subsequently embedded in paraffin and sectioned at 3 to 6 microns.

Both whole mounts and sections were stained with azure B and eosin or with hematoxylin-azure-eosin for morphological study. For differential staining of both nucleic acids an azure B bromide solution adjusted to pH 4.0 was used, the Jenner-Giemsa stain of Jacobson and Webb (16) was applied to whole mounts, and methyl green–pyronine staining was also carried out. For the latter, methyl green was purified according to the directions of Pollister and Leuchtenberger (17) and either recrystallized pyronine B, or pyronine Y as recommended by Kurnick (18) were used. To exclude the possible interfering effects of the amino groups of associated protein on basic dye binding by RNA, preparations were acetylated in absolute acetic anhydride at room temperature for 2 hours before staining with azure B. Inactivation of protein-bound amino groups for the same purpose is effected by formalization, or by nitrosation in the case of Love's differential staining method for RNA (19) which was also applied.

Differential localization of both RNA and DNA in infected cells was also studied by means of induced fluorescence in preparations stained with the fluorochrome acridine orange, 0.1 per cent at pH 3.6 for 15 minutes or 0.01 per cent at pH 3.6 for 30 minutes. The material was observed in a Zeiss fluorescence microscope fitted with a cardioid darkfield condenser. Exciting long ultraviolet radiation was isolated from A Zeiss osram BHO 200 W high pressure mercury arc lamp through filter BG 12 (transmission peak, 425 mμ) and Corning filter 7-59 (transmission peak, 355 mμ). DNA was identified by means of the Feulgen reaction, using hydrolysis times (in .10 N HCl) of 7 to 8 minutes for methanol-fixed monolayers, 12 minutes for formalin and for alcohol–chloroform–trichloracetic acid-fixed sections, and 18 minutes for osmium tetroxide–fixed sections.

Control preparations for selective removal of RNA were digested with 0.05 per cent crystalline ribonuclease in doubly distilled water at 37°C. for periods of from 30 to 60 minutes. Both nucleic acids were extracted by treatment with 5 per cent trichloracetic acid at 90°C.

for from 15 to 45 minutes. Specific enzymatic removal of DNA was attempted with 0.025 per cent crystalline deoxyribonuclease in a 0.1 per cent gelatin solution containing 0.03 M $MgSO_4$ at 37°C. for 1 to 3 hours.

Proteins were identified by the following group reactions. The coupled tetrazonium reaction for phenolic and heterocyclic residues was carried out as prescribed by Pearse (20) using *o*-dianisidine as the tetrazotate. Acetylated controls were used as checks. The cytochemical Millon reaction for tyrosine residues was employed with the modifications suggested by Rasch and Swift (21). Arginine residues were detected with a modification of Baker's version of the Sakaguchi reaction (22) in which good results were obtained by omitting the pyridine-chloroform step of the former. Tryptophan was studied with the histochemical test of Glenner and Lillie for indolic residues (23). Alpha (terminal) amino groups of protein were demonstrated with the ninhydrin–Schiff reagent method of Yasuma and Ichikawa (24).

To gain some information about the relative concentration of protein all available basic (amino) groups, including also those of the lysine, arginine, and histidine residues were stained with naphthol yellow S (flavianic acid) as elaborated by Deitch (25), or with the anionic dye fast green FCF in acetate buffer at pH 2.5 (26). Acetylation of sections in absolute acetic anhydride at room temperature (25°C.) for periods up to 2 hours (25–27) was used as a control to block the amino groups.

Basic protein of the histone type was localized by means of alkaline fast green staining at high pH after extraction of nucleic acids, as detailed by Alfert and Geschwind (28). In instances in which the distribution of both Feulgen-stainable DNA and histone were to be studied in the same object the modified Feulgen procedure of Bloch and Godman was used (29). The presence of protein-bound sulfhydril and disulfide groups was revealed in cells fixed in fluids containing trichloracetic acid with the DDD method of Barrnett and Seligman (30) and the use of suitable control sections treated with iodine, *N*-ethylmaleimide, or iodoacetic acid.

Extractions or digestions were used to assist in characterizing certain materials in sections of "frozen-substituted" cells. These extracting agents included M NaCl, 5 per cent HCl, M/15 phosphate buffers, 20 per cent urea at 25°C. for 30 to 60 minutes and salt-free trypsin (0.01 per cent, pH 7.8) and pepsin (0.2 per cent, pH 1.6) at 37°C. for 1 to 3 hours.

As another means of characterizing protein structures in the absence of nucleic acid, their affinity for acid and basic dyes at various pHs was determined by staining sections of methanol-fixed or frozen-substituted cells in 5×10^{-4} M solutions of methylene blue or orange G in a series of buffers of low ionic strength in graded steps of 0.5 units from pH 6.5 to 2.0 (31).

The PAS reaction was applied to identify components with vicinal hydroxyl or aminohydroxyl groupings. Lipid was sought in formalin-fixed whole mounts by staining with Sudan black B dissolved in 60 per cent triethylphosphate.

In many instances, the same bodies identified in azure-eosin, azure, or Feulgen-stained preparations were subsequently subjected to a successive series of tests for identification of components. Such sections were mapped photographically to assist identification.

OBSERVATIONS

Healthy control cultures of both HEp-2 (FJ) and HeLa lines grew in sheets. The cells showed the polymorphism and variation of nuclear and nucleolar size often described, but the nuclei of the predominating cell type averaged 18.3 ± 3.6 μ in diameter. Mitotic figures were very numerous, with some abnormal forms always present. In from 10 to 50 per cent of some otherwise healthy appearing cells an intensely acidophilic granular body of 10 to 15 μ diameter was

seen in the juxtanuclear cytoplasm surrounded by a clear halo. The presence of this body or bodies, evidently composed of protein appeared to have no effect on the selection of cells by the adenovirus for infection, or on the progress of the resulting nuclear lesion.

After infection the cell sheet or monolayer showed a disposition to focal cell loss by clumping as the pathological changes became widespread, but retraction of whole sheets was unusual. Infected cells tended to become rounded, and, in late stages, to be washed off the coverslip surface. Groups of contiguous or neighboring cells generally tended to be affected, with the formation of small islands or plaques.

Cells of both HeLa and HEp-2 (FJ) fixed 2 to 3 days after infection with type 5 adenovirus exhibited a variety of altered appearances which changed as the lesions progressed.

At first, up to 24 hours, only about 7 to 10 per cent of the total population of cells was visibly affected. After 24 hours an increasing proportion of cells exhibited characteristic pathological changes with time, and by 72 hours, 75 to 80 per cent of the cells adhering to the glass showed well marked signs of infection. Until 36 hours, the stages of infection developed with remarkable synchrony; thereafter the stages were increasingly out of phase, presumably owing to the appearance of early lesions in newly infected cells, and to possible variability in the rate of development of changes which had been initiated at the time of infection. Nevertheless from 36 to 72 hours after inoculation the lesions resulting from the first cycle of infection predominated.

Four stages have been arbitrarily chosen from a continuous sequence of pathological change. The predominating or characteristic form appearing in the infected cell sheet during any time interval will be described; however at any time after stage 2 smaller numbers of cells in other stages were also present. As in the case of all the adenovirus types studied, the changes affect chiefly the cell nucleus, which will be described in detail.

Morphology:

Stage 1, 12 to 20 Hours after Inoculation.—The first discernible structural change was the occurrence of acidophilic granules (from 0.5 to 1.5 microns in size) either dispersed, or collected into small groups, clusters, or rows (Fig. 1, *a*). At higher resolution minute vesicles or rings of about 0.5 μ diameter could be discerned amidst the acidophilic material. The chromatin remained dispersed and exhibited its usual tinctorial properties, and the nucleoli remained unchanged in form, number, location, and stainability. Mitotic figures continued to be numerous in uninfected cells of the cultures up 20 hours after inoculation.

Stage 2, 20 to 26 Hours after Inoculation.—Greater numbers of nuclei were affected by this time. The acidophilic granules were increased in number and further agglutinated into clusters and masses (Fig. 2), which somewhat later had joined (Fig. 3) and partly coalesced toward the center of the nucleus, forming either a loose acido-

philic network or large masses (Figs. 4 to 6). In many nuclei of this stage, within the granular acidophilic material, sharply outlined round, prolate, or elliptical bodies measuring from 1.0 to 4.0 μ were to be found (Figs. 3 to 6, *b*). They usually had the appearance of discrete rings or vesicles with sharp marginal lines which were found either "empty" (Fig. 6, *b*) or more often enclosing numbers of coarse acidophilic granules and more homogeneous acidophilic material. These limiting rims or membranes sometimes appeared to be open or incomplete at one end, giving the structure a crescentic or parabolic outline; in some instances the membranes were doubly contoured.

Continued fusion of granules and clusters resulted in a denser more compact appearance of the acidophilic material disposed into interconnected cords and trabeculae (Fig. 6, *a*).

Nuclei at this stage of infection were almost always enlarged in moderate to marked degree (mean diameter 25 $\pm$ 7.4 μ). They seemed to be swollen by additional contents which filled the space between the granular masses and the membrane resulting in the occurrence of a relatively wide, apparently clear space or halo between the distinctly delineated nuclear membrane and the aggregated acidophilic material gathered toward the center of the nucleus.

The chromatin, now reduced and displaced, lay among and between the cords of acidophilic material or in a few irregular strands bridging the space between the nuclear center and the inner aspect of the membrane. The nucleoli remained undisturbed throughout stage 2.

In cultures at this stage there was a reduction in the number of mitotic figures to about ½ to ⅔ of that in infected controls.

Stage 3, 24 to 30 Hours after Inoculation.—With the formation of the trabecular mass or aggregate in stage 2, small intensely basophilic granules were then found deposited upon or within the acidophilic cords. By stage 3, this basophilic granulation or stippling of the condensing trabeculae of the central mass had become prominent (*cm* in Figs. 7 and 9). This basophilic material was found in discrete granules disseminated throughout the acidophilic central mass or agglomerated into small masses or filaments (Fig. 7). The amount of basophilic material increased throughout stage 3, but varied considerably in quantity in different nuclei at this stage of the lesion: in some only a few scattered basophilic granules could be seen in a relatively "mature," *i.e.* condensed, central acidophilic mass (*cf.* Figs. 9 and 10). The latter continued to become more compact. The sharply contoured vesicular bodies were now less numerous than in stage 2. Nuclei at this stage were more enlarged, and their chromatin distribution distorted. This could be observed best in preparations stained for histones. Small, round, dense nucleoli were still often present within the affected nuclei.

With increased formation of basophilic material, the acidophilic material of the central network or mass underwent still further condensation, fusion, and homogenization of texture. The space between the trabeculae of the mass, like the space between it and the nuclear membrane gave the impression of being filled with a slightly refractile substance (Figs. 7, 8, *m*). The continued accumulation of this substance expanded the intertrabecular spaces into distended vacuoles or locules, compressing the acidophilic material and distending the nuclear membrane. The formation of the refractile substance and its retention in the nucleus undoubtedly had been proceeding during stages 1 and 2 as evidenced by nuclear enlargement (Figs 3 to 5), but it was

not demonstrable at early stages in fixed preparations presumably owing to its solubility and its chromophobia. Late in stage 3, however, when it was very abundant, it could be seen as a diffuse homogeneous refractive vitreous or somewhat opalescent material, which usually accepted anionic dyes but slightly. Floccules, granules, or areas of greater density which were sometimes present within the otherwise glassy or hyaline substance (hereafter designated "matrix") were more acidophilic (Fig. 21). The contoured, sharply outlined, vesicular bodies of earlier stages were evident only rarely in stage 3 (Figs. 6, 9). Small nucleoli persisted in many of the nuclei of this stage. The basophilic masses tended to increase and coalesce. At this time the affected nuclei were usually greatly enlarged and deformed. Only an estimated 10 to 15 per cent of the cells of the sheet showed these cytopathologic changes at 24 to 36 hours in most specimens. Mitotic figures were reduced as in the previous stage.

Stage 4, from 30 Hours.—The accumulation and the condensation of the hyaline matrix was accompanied by crystallization within it of asymmetric crystalline bodies. These slender elongate rodlets with straight sides and squared or angular faces were at first quite small (2 to 4 microns in length) and could be found within the locules of the central mass and in the zone between nuclear membrane and central mass (Figs. 9, 10, 14). Later development entailed marked growth of these crystals in size and number, so that ultimately the larger ones, which measured up to 40 microns, filled, sometimes stretched, and further distorted the already distended nuclear envolope (Figs. 15 and 16). They were moderately acidophilic and strongly refractile in mismatched media, and gave the altered cells a striking and characteristic aspect. In most cases the central mass was no longer a loculated or vacuolated network but had further contracted into a tight, dense rounded body whose acidophilic component was usually homogeneous (Figs. 15 and 16). Within this body, which lay centrally or somewhat eccentrically in the nucleus, often touching the nuclear membrane, there was a variable amount of basophilic material. In most instances it came to occupy all or most of the central body which then appeared as an intensely basophilic ball with an acidophilic periphery; in other nuclei the basophilic material in the form of granules and filaments was laced through the condensed acidophilic substance (Fig. 10, *f*). In occasional nuclei of this stage, relatively little basophilic material was formed or had remained within the acidophilic central mass. From the central mass occasional slender strands or partitions bridged the wide space containing the amorphous matrix to the inner aspect of the nuclear membrane, to which they appeared to be attached. At these points of attachment the nuclear membrane was usually puckered or retracted, while the intervening stretches were ballooned out as though by the turgor of the nuclear contents. This appeared to divide this zone into compartments, a circumstance which, with the increase of the vitreous matrix, gave rise to the arresting rosette or flower-like forms of late stage 4 (Figs. 14 to 16). In some instances the glassy matrix, usually only slightly acidophilic, appeared to be of greater density and concentration, as though inspissated, and stained fairly intensely with anionic dyes (Fig. 13). Such areas were devoid of crystals. Later in stage 4, in many instances, the amorphous matrix material acquired a diffuse basophilia and was found heaped about the large crystals as sleeves or cuffs or casings (Figs. 15, 18). The enlarged nuclei averaged $32 \pm 8.8\ \mu$ in diameter.

Stage 4 appears to persist for an unusual long period; from 48 hours until after 96 hours it was the most frequently observed stage, in spite of the tendency of cells with advanced cytopathogenetic changes to fall off the coverglass. After 48 hours, most inoculated cultures show widespread morbid changes associated with adenovirus infection, from 25 to 60 per cent of cells counted exhibiting some stage of the lesion. Mitotic activity remained reduced. In this connection it is noteworthy that the changes characteristic of stage 4 were on rare occasions seen in mitotic cells containing well formed chromosomes, presumably of prophase or arrested metaphase (Fig. 17).

Histochemistry:

1. The Acidophilic Granules, Aggregations, and Central Trabecular Mass of Stages 1 to 3.—Acetylation with acetic anhydride or deamination with nitrous acid of most of the basic groups upon which acid dye binding depends, markedly reduced the acidophilia of this material (25–27, 31, 32). Digestion with trypsin and pepsin caused partial disintegration of the central masses and rendered them unstainable. Urea and 1 M NaCl caused marked swelling and loss of dye affinity.

Group reactions for protein: Uptake of naphthol yellow S at pH 1.0 as indicated above, revealed the amino groups of protein in lysine, arginine, and histidine residues, and the terminal (alpha) amino groups; the latter were also shown in the moderately colored ninhydrin–Schiff reaction of Yasuma and Ichikawa (24). The presence of cyclic amino acid residues was suggested by the strongly positive coupled tetrazonium reaction, and confirmed the modified cytochemical Millon test for tyrosine, which showed the aggregates as moderately colored. Arginine residues were revealed in the cytochemical Sakaguchi reaction, which was moderate in the aggregated masses, and somewhat more intense in the recognizable marginated and redistributed chromatin. The distribution of the latter could also be shown to advantage by use of the alkaline fast green stain for basic proteins of the histone type. The acidophile granules or reticular aggregates did not accept this stain at all. The remaining histone-containing chromatin material which was stained, although apparently reduced in amount, could be seen in stages 3 and 4 to be distributed marginally against the nuclear membrane, and to surround the central reticular mass, some of the vesicular bodies, and the nucleoli, as delicate rims. The aggregates stained very faintly in the Barrnett-Seligman reaction for both sulfhydril and disulfide groups (30); appropriate controls were unstained.

Nucleic acids: With azure B bromide at PH below 4.2 the acidophile granules and aggregates were faintly stained a dull lavender color. Predigestion with ribonuclease for up to 1 hour had no effect on the hue or intensity of staining. With the methyl green-pyronine stain this material appeared red to faintly greyish-pink, almost chromophobic. The material of these aggregates did not stain with the Feulgen reaction (Figs. 11, 12, 19, 20).

Acetylation to block basic protein groups which might interfere with the uptake of basic dyes by nucleic acid, did not result in augmented basophilia or metachromasia in the aggregates of stages 1 to 3, nor was nucleic acid demonstrable in the granules or aggregates with Love's method, in which inactivation of protein amino groups is also followed by a basic stain (19). With acridine orange as fluorochrome for differential detection of nucleic acids, no reddish fluorescence denoting the presence of RNA (33) was observed within the nuclei at any stage of infection.

Carbohydrate and lipid, as detected in the PAS test, and by Sudan black B staining after fixation in neutral formalin were not found in these bodies.

2. The Basophilic Bodies of Stages 3 and 4.—These were readily visualized with any basic dye and were stained selectively with basic dye in mixtures of cationic and anionic dyes, as azure-eosin. More prolonged incubation of sections with trypsin (2 to 3 hours) and pepsin was necessary to effect their removal than was necessary to digest the acidophilic reticular aggregates or central masses within which they lay. They stained like DNA with methyl green–pyronine and the Jenner-Giemsa and were strongly colored in the Feulgen reaction (Figs. 12, 18, 22). Pretreatment with ribonuclease did not affect their stainability. More vigorous extraction with 5 per cent trichloracetic acid at 90°C. was usually necessary to extinguish both basophilia and the Feulgen reaction of these bodies than was required for eliminating the stainability of chromatin. Digestion of methanol-fixed cells for 1 hour with deoxyribonuclease abolished basophilia and Feulgen stainability of both chromatin and these granules in some but not all experiments. The granules, however, were more often resistant to the action of the enzyme.

Protein: The basophilic granules stained more strongly in each of the group reactions for protein than did the background material of the central or reticular masses. After extraction of nucleic acids with hot trichloracetic acid, they displayed more affinity for anionic dyes than the background material in which they were embedded. They exhibited intense coloration after the Sakaguchi reaction for arginine (Fig. 25).

Carbohydrate and lipid were not found in them.

3. The Matrix and the Asymmetric Crystals of Stage 4.—The substance (*i.e.* "matrix") collected between the central mass and the nuclear membranes and in the vacuoles of the central mass became apparent in fixed preparations after it had become sufficiently concentrated to be visible as a hyaline material, at first chromophobic or very faintly acidophilic and subsequently somewhat more acidophilic. The condensed or inspissated amorphous matric material found in some nuclei at stage 4 (Fig. 13) had the same qualitative properties as the asymmetric crystals. The crystals and condensed matrices were acidophilic. They were easily digested with both trypsin and pepsin acting for relatively short periods (30 to 45 minutes); urea and M NaCl caused them to swell markedly. Dilute alkali but not acid caused their swelling and dissolution. Their extinction point for methylene blue–staining was higher than pH 5.2 in contrast to that of chromatin and the basophilic granules, which was below pH 2.6.

Protein group reactions: Acetylation reduced but did not extinguish the affinity of matrix and crystals for naphthol yellow S, suggesting that other than the ϵ-amino of lysine residues were contributing to the acidophilia. The crystals stained faintly to moderately in the ninhydrin–Schiff test for α-amino acids. The group reactions for cyclic amino acids (Fig. 24) and for tyrosine stained the crystals strongly (Fig. 23), but in no case so intensely as the basophilic bodies within the central mass. The reaction for indolic residues, however, was very faint as was the test for sulfhydril-disulfide groups. Arginine residues were not detectable in the crystals with the cytochemical Sakaguchi reaction (Fig. 25) nor did they stain with the alkaline fast green.

Nucleic acid: Crystals and condensed matrix gave none of the reactions for either nucleic acid, using the methods listed above, (Figs. 18, 21, 22). Later in stage 4, the dilute matrix material often became diffusely or focally basophilic, the area so stained

being poorly demarcated and most often seen surrounding the crystals. This blue-purple azurophilia of this material was unaffected by ribonuclease. It was stained with the Feulgen reaction (Fig. 18) moderately with methyl green, and was extractible with hot trichloracetic acid.

In Fig. 27 the same crystal, identified in the electron microscope in Fig. 26, is shown in adjacent thick sections to be Feulgen-negative, but stainable with acid fast green which is bound to protein basic groups.

Carbohydrate and lipid were not detected in either the matrix or the crystals.

DISCUSSION

I. Structural.—Morphological studies by Barski (1), Barski and Cornefert (6), Boyer, Leuchtenberger, and Ginsberg (4) and especially Boyer, Denny, and Ginsberg (9) have established that the first seven serotypes of adenovirus are divisible into two groups, 1, 2, and 5 and 3, 4, and 7, on the basis of differences in the character of the lesions which they bring about in cultured cells. Members of each group which appear to exhibit similar growth cycles and quantitative neutralization reaction with specific antibody (34) also produce a similar cytopathological effect on a variety of different cell lines including HeLa, human, simian, and rabbit kidney, rabbit spleen and fibroblast, human amnion, and also HEp-2 cells.

The cytomorphological changes following type 5 adenovirus infection have been studied in a number of laboratories (5, 6, 8, 11–14, 35, 36 *inter alia*); like types 1 and 2, the virus has been found to provoke the formation of eosinophilic, subsequently basophilic, polymorphous, intranuclear bodies and a marked degree of vacuolization of nuclei in contrast to the more compact intranuclear bodies of types 3 and 7 (6, 12). The late lesion of type 5 infection apparently differs from that of the related types 1 and 2 in the occurrence of non-viral protein crystals in the vacuolar areas of the stricken nuclei. These were first pictured in unfixed infected cells photographed with the interference microscope by Leuchtenberger and Boyer (14), as bar-like crystals. Subsequently Morgan *et al.* (5) distinguishing between viral aggregates non-viral protein crystals revealed the identity and characteristics of the latter by means of electron microscopy correlated with histochemical methods. Although these crystals have been represented in the description of cells infected with strains of type 5 adenovirus by Leuchtenberger and Boyer (14), Morgan *et al.* (5), Nasz and Toth (11), and Boyer, Denny, and Ginsberg (12) they are evidently not a consistent feature of infection with all strains of type 5 adenovirus and were not mentioned in the reports of Barsky (1), Barski and Cornefert (6), Prier and LeBeau (8), Pereira, Allison, and Balfour (13), Lagermalm *et al.* (35), and Fukumi *et al.* (36). Although in our studies these crystals were somewhat less frequent and smaller in HeLa and amnion cells than in HeP-2 cells, using the same viral strains (AD-75 and No. 196) they were consistently found. Their formation, according

to Boyer, Denny, and Ginsberg (12) is a property related to the strain of type 5 virus, rather than to the host cell line.

These authors have hardly distinguished between the granular or fused aggregates of acidophilic material and the spheroid rimmed or vesicular structures, with or without cores, which they and others (4, 11) have described. In our material, and apparently also in that of Nasz and Toth (11), diffuse intranuclear acidophilic granules and clusters appeared to precede the formation of the rimmed or contoured bodies, nor were the latter invariably present in cells containing eosinophilic aggregates. The actual significance of these contoured bodies is unknown. In the nuclear lesions produced by a given strain, considerable variation in the amount and distribution of particulate basophilic material, was found from cell to cell in comparable stages of development, suggesting that there is little quantitative uniformity in the synthesis of virus.

The decline of the mitotic indices of infected cultures parallels the extent of the cytopathic effect, and at least in part is a reflection of the increase in numbers of infected cells that cannot divide. The rare occurrence of cells with the stigmata of late stages of infection in which well formed chromosomes are found (Fig. 17) is a mere curiosity. There was no evidence to suggest that infected cells could complete mitosis.

II. Histochemical.—From the results of this qualitative histochemical survey, it can be concluded that the aggregates and the reticular or central masses formed from them consist of a non-histone protein in which, at first, neither nucleic acid is detectable. A negative test does not permit the conclusion that a given substance is absent; since, however, the Feulgen reaction for DNA is quite sensitive, it is improbable that polymerized DNA is present in these masses in stages 1 and 2. Neither was RNA detectable, but the presence of RNA in concentration too small to bind significant amounts of basic dye cannot be ruled out.

Ribonucleic acid has not been mentioned as a cytochemically apparent constituent of the infected nucleus by other investigators employing differentially fluorescent acridine derivatives for simultaneous detection of both nucleic acids and discrimination between them (10). In these investigations competitive interference effects and insufficient sensitivity may render it impossible to demonstrate small concentrations of RNA in the presence of much protein, and therefore the possibility that RNA may be present in the infected nucleus cannot be rigorously excluded. The basophilic granules, filaments, and masses of stages 3 and 4 are plainly composed of deoxyribonucleoprotein, each moiety of which is highly concentrated as judged by intensity of the staining reactions and the density of the bodies. A noteworthy feature of the DNA of these granules and masses is its greater resistance, relative to chromatin DNA, to extraction by hot trichloracetic acid, a property probably resulting from a closer or firmer link of viral DNA and its protein than that of chromatin. Armstrong

and Hopper (10) have recorded that the DNA of the basophilic bodies of type 6 adenovirus infection is resistant to depolymerization by DNase unless previously digested with a protease. The protein, which is itself somewhat more resistant to proteolytic digestion than chromatin or cytoplasm, is a "higher" protein of the non-histone type with a content of arginine residues. Since the evidence indicates that this nucleoprotein is viral, it is pertinent to recall the well-known difficulty of separating viral nucleic acid from its protein and the resistance of intact viruses to nucleases (10, 37–39). The "matrix" and the crystals associated with it are a non-histone protein, without other detectable organic components. This protein is characterized by ready digestibility, a relatively high tryosine, and low or absent arginine content. As judged by its colorability this protein is less concentrated than that of the acidophilic masses or the viral nucleoprotein granules. The protein of the matrix material of stages 1 to 3 is evidently more difficult to fix, more soluble after fixation, or highly dilute; in later stages it can be stained as a homogeneous material exhibiting qualitative properties similar to those of the crystals. Late in the development of the lesion, the "matrix" comes to contain large amounts of DNA which, as the electron microscope shows, reflect the presence of numerous scattered viral particles and not properly "matrix" *per se.*

It thus appears that in its solubility and digestibility, in the absence of Sakaguchi-stainable arginine and fast green–stainable basic protein, the protein of the non-viral crystals differs qualitatively from that of the acidophilic material of stages 1 and 2, from the protein of the basophilic (viral) granules and masses, and from the chromatin of the nucleus.

III. Correlative.—Electron microscopic examination of thin sections permits only limited sampling, for reasons which have been discussed elsewhere (40), and tells little about the chemical composition of structures. When electron micrographs, however, are correlated with the temporal and topochemical information gathered by light microscopy, it becomes possible to reconstruct many of the events in the intranuclear development of type 5 adenovirus and to show the evolution of these changes.

The most satisfactory correlation is obtained when the same cell is examined in contiguous thick and thin sections by light and electron microscopy, respectively. In Fig. 26, for example, the electron micrograph clearly illustrates a non-viral crystal, which the photomicrographs (Figs. 27 a and b) show is protein in nature and devoid of deoxyribonucleic acid. In order to assess the temporal sequence of changes, however, and to determine in so far as possible the chemical nature of the structures observed, it was found advantageous to employ whole cell mounts to which a wide variety of histochemical procedures could be applied. It was then relatively simple to fit the electron micrographs into the stages of development and to ascertain the fine structure of the principal nuclear components.

The first alteration visible in the light microscope is the deposition of the acidophilic granules shown in Fig. 1. These undoubtedly correspond to the reticular aggregates encountered in the electron microscope before appearance of recognizable virus, as illustrated in Fig. 2M.[1] Subsequently, in stage 2, the granules increase in number, enlarge and become more dense (Figs. 2 and 4 and 4M), often coalescing to form strands (Figs. 3 and 3M). In these early stages the acidophilic material consists predominantly or entirely of protein and has no detectable nucleic acid. The immunohistochemical investigations of Boyer, Denny, and Ginsberg (12) and Pereira *et al.* (13), however, revealed specific fluorescence with type-specific antibody and it must therefore be assumed that the material contains type 5 viral antigen. This observation, together with the close spatial relationship which the viral particles and reticulum exhibit (Figs. 10M and 11M) and the fact that as the former increase in numbers the latter diminishes in amount, strongly suggest that the virus differentiates from the reticulum. Presumably the process is rapid, for stages in the development of individual viral particles are difficult to identify with any degree of consistency. The question naturally arises, then, as to why histochemical methods fail to demonstrate nucleic acid in the reticulum. It must be assumed either that it is there in insufficient concentration to demonstrate, or that soluble precursor materials, rather than the fully polymerized nucleic acid, are held at this site.

When the reticular aggregates condense and assume a central position within the nucleus (Figs. 7 to 9, and 9M and 12M), large numbers of viral particles have generally formed. Collection of virus upon or close to the trabeculae of reticular material is correlated with the appearance of basophilic granules and cords in the central masses of the photomicrographs (Figs. 7 to 9). This deoxyribonucleoprotein material, as demonstrated by Feulgen staining (Figs. 12, 18 and 22), is evidently composed of small crystals and agglomerations of viral particles, similar to those encountered in studies of type 3 adenovirus (3). Although the possibility that some of the DNA may lie outside the viral particles cannot be excluded, the nucleic acid has properties which might be expected of the firmly linked viral nucleoprotein. Both nucleic acid and protein are in high concentration, and, as has been indicated, this protein moiety is histochemically distinguishable from both chromatin and "matrix" material. The granules were found by Boyer *et al.* (12) to be the site of brilliant fluorescence after exposure to labeled antibody, indicating that the protein contains viral antigen.

The intertrabecular and peripheral zones of diminished density in which the non-viral protein crystals form (Figs. 6M to 8M) represent the amorphous hyaline or glassy matrix seen in Figs. 7, 8, and 21. The crystals, when they

[1] The designation M refers to electron micrograph figures in Paper I.

achieve sufficient size to be recognized in the light microscope, show the characteristic hexagonal faces (lower left of Fig. 23) seen in cross-section in the electron microscope (Figs. 12M, 16M, and 26), and the elongated outlines encountered in longitudinal sections (compare Fig. 10 with 25M and Fig. 24 with 21M). These remarkable crystals, like the matrix material, have been shown to consist of protein devoid of nucleic acid (5), and fail to bind fluorescent type-specific antibody (12). Viral particles may be found dispersed in the matrix, where they are revealed by the electron microscope, even before stage 3. The number of disseminated viral particles often reaches a concentration sufficient to confer a distinct, diffuse basophilia, and Fuelgen stainability on the matrix (Fig. 18). This unaggregated virus occasionally becomes heaped around the large non-viral protein crystals, giving the appearance of a basophilic and Feulgen-stained cuff (Figs. 16 and 18). Such disposition of virus may be equated with the streaked fluorescence of labeled antibody found by Boyer *et al.* (12) alongside the non-fluorescent bars (*i.e.*, non-viral crystals). In addition, numerous viral particles continue to lie within the condensed central reticular mass. The components in the electron micrographs illustrated by Figs. 12M and 21M may be matched with the photomicrographs showing advanced stages of infection (Figs. 15, 16, and 18): the scattered virus in the matrix as diffuse basophilia, the viral crystals occupying much of the condensed, reticular material (eccentrically placed in Fig. 21M) as intense basophilic, Feulgen-stained granules or masses, and the elongated crystals (cross-sectioned in Fig. 12M) as protein-containing, Feulgen-negative structures.

The vesicular or contoured bodies with limiting rims encountered in stages 2 and 3 (marked *b* in Figs. 2, 4 to 6, and 9) probably correspond to the sharply defined homogeneous bodies or vesicles with thick electron-dense walls enclosing fine reticular contents (Figs. 2M, 9M, 12M, and 30M). Whether the homogeneous bodies actually represent vesicular structures sectioned eccentrically through their wall is not known. Although studies with fluorescent antibodies have revealed type-specific antigen localized in the rims of these bodies, electron microscopic examination did not reveal any consistent spatial orientation of the vesicles to the sites of viral differentiation. The possibility that the small, dense, homogeneous granules closely applied to the reticular material in Figs. 10M and 16M represent aggregates of antigen, some of which may increase in size and form vesicles, cannot be excluded, but structures that could be interpreted as forms in the process of this transition were rare.

Nucleoli did not seem to participate in the production of virus for they appeared little altered in appearance despite advanced nuclear changes (see Figs. 1 to 9 and 14, as well as 12M, 13M, and 17M). The striking granularity occasionally observed (Figs. 14M and 15M) has been seen in uninfected, control preparations and, hence, cannot be ascribed to any specific action of the virus.

The persistence of cells with unbroken nuclear membranes enclosing masses of virus at late stages of infection is noteworthy. This observation corresponds well with the facts reported by Ginsberg (41) and suggested by Lieberman and Friedman (42) namely, that during the incremental period appreciable amounts of newly synthesized virus were not released into the fluid phase of the culture, and even several days after inoculation spontaneous release of virus could only account for about 5 per cent of the total recoverable virus. The presence of virus in the cytoplasm appears to be consequent upon nuclear disruption (Fig. 17M) and release of virus upon dissolution of the cell (20M and 21M), in striking contrast to the mechanisms by which herpes simplex virus seems to gain egress from intact cells (43).

Pathognomonic changes were not noted in the cytoplasm either by light or electron microscopy.

IV. General.—Cytochemical methods are of special value in defining the nature of the different bodies described not only because they permit selection, out of a heterogeneous population, of a cell in any given stage, but also because they allow study of particular structures within a nucleus containing several different inclusions. Among the methods at hand for cytochemical characterization of protein, application of the fluorescent antibody method of Coons is most useful. Immunohistochemical (fluorescent antibody) studies by Boyer, Denny, and Ginsberg on the intranuclear bodies in type 4 (9) and type 7 (12) adenovirus infection have provided valuable data for comparison with the localization of antigen after type 5 infection (12, 13). The acidophilic bodies which form in the nuclei in earliest stages of infection with adenovirus types 4 and 7 evidently contain no homologous type-specific antigen (9, 12), while as has been noted, those of type 5 infection do exhibit specific fluorescence (12, 13). Such antigen, in the case of type 4 or 7 infection appears only with the basophilic, presumably viral, masses to which it remains localized. In these cases fluorochrome-stainable antigen is absent from the peripheral zone of infected nuclei, as it is from the "matrix" and crystals of type 5 infection (12). If viral antigen is indeed absent from the latter structures, it would militate against the hypothesis that "matrix" and crystals are essentially an excess of viral protein analagous to the non-infective protein of plant virus (44).

Adenovirus infection stimulates augmented P^{32} uptake into infected cells (45–47). In both type 2 and type 5 infection, increased labeling with P^{32} was found in the RNA and DNA fractions, with additional, more prolonged uptake into DNA (47). It has been proposed that this enhanced uptake into RNA in type 5 infected cells is a reflection of augmented RNA synthesis in the production of a specific cytotoxic material, which has been shown to be a ribonucleoprotein complex (48, 49). It has also been contended by Tamm and Osterhout (50) that RNA has a necessary part in the synthesis of DNA virus, chiefly on

the basis of the observation that dichlororibofuranosylbenzimidazole, an inhibitor of RNA synthesis, inhibits multiplication of type 4 adenovirus and influenza B virus. Cytochemical methods have not been conclusive in estimating a change in RNA concentration in infected cells, nor has RNA been definitely localized in infected nuclei outside the nucleoli.

Quantitative cytochemical and biochemical studies showing some increase in the total amount of DNA in cells infected with adenovirus types 1, 2, 3, and 4 have been more definitive (4, 45–47). Although the base ratios of P^{32}-labeled DNA isolated from cells infected with type 2 adenovirus are not different from that of uninfected cell DNA, its guanylic and cytidylic deoxyribonucleotides apparently have consistently higher specific activities than those of its deoxyadenylic acid (47). This suggests that a new kind of DNA is being synthesized. According to Ginsberg and Dixon (46) some 25 per cent of the DNA extracted from cells infected with type 4 adenovirus, which reportedly also differs from normal host DNA in its solubility properties, is not incorporated into infectious virus particles and is supposedly therefore newly synthesized. Cytochemical measurements now being made of the relative amounts of both DNA and protein in infected cells at each stage in the sequence of pathological alteration should begin to provide data on the synthesis of DNA in relation to viral development especially in the eclipse or preparticle phase.

A microspectrophotometric study of similar kind, carried out on infected cells of the viral papilloma of human skin showed that increased amounts of DNA are synthesized before or at the time of the earliest morbid cytological change (appearance of an acidophilic inclusion body) and that DNA does not increase appreciably in amount of thereafter, although it is relocated, presumably into virus, only at a later stage (51). The virus of the infectious papilloma of human skin, like the adenoviruses, is a crystallizing particle which develops intranuclearly (52). Like the adenoviruses, it produces a sequence of cytopathologic changes in which a protein inclusion body devoid of detectable DNA first appears, followed in turn by the development of a DNA-containing mass, presumably viral. It remains to be seen whether the pattern and sequence of DNA synthesis is also similar in the case of the adenoviruses.

SUMMARY

The evolution of the intranuclear lesion produced by type 5 adenovirus in HEp-2 and HeLa cells is described as seen in the light microscope and the bodies formed in the course of the infection characterized histochemically. Some 12 hours after infection acidophilic protein bodies, without appreciable nucleic acid, first appear in the nucleus and coalesce into a network. Within or in association with this material, DNA-containing masses (viral aggregates) are formed which rapidly increase in amount and then coalesce. At the same time, a protein

is produced, histochemically different from that of the acidophilic or basophilic structures mentioned, within the infected nucleus, which constitutes a matrix within which regular cytstals of a protein, (presumably non-viral) materialize. These structural and histochemical features are correlated with details which have been observed in parallel studies with the electron microscope.

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EXPLANATION OF PLATES

PLATE 33

All figures are of whole mounts stained with azure-eosin.

FIG. 1. Nucleus of HEp-2 cell 18 hours after infection, showing changes characteristic of stage 1. The nucleus is enlarged, and accumulations of granules and bodies of acidophilic material are shown at *a*. The nucleoli (*n*) are intact. × 1550.

FIG. 2. Nucleus of HEp-2 cell 20 hours after infection, stage 1. The acidophilic material is aggregated into large solid clusters and bodies (*a*). × 1550.

FIG. 3. Nucleus of HEp-2 cell 20 hours after infection, stage 2. Ring-like or oval "vesicular" bodies, with sharply outlined rims (*b*) are present within the granular clusters and masses of acidophilic material. × 1550.

FIG. 4. Nucleus of HEp-2 cell 22 hours after infection, stage 2. The "vesicular" sharply contoured bodies shown at *b* appear to have moderately dense contents. They lie in a compact mass of granular acidophilic material (*a*). × 1550.

FIG. 5. Nucleus of HEp-2 cell 24 hours after infection, stage 2. Abundant compact mass of acidophilic material (*a*) aggregated toward center of enlarged nucleus. Within it are the rimmed "vesicular" inclusions (*b*). × 1480.

FIG. 6. Nucleus of HEp-2 cell 32 hours after infection, stage 2. Some connected vesicular bodies (*b*) with sharply outlined rims are shown. The borders or rims have the appearance of membranes. The contoured bodies lie within a trabecular network of acidophilic material (*a*) which encloses locules appearing as clear spaces. × 1300.

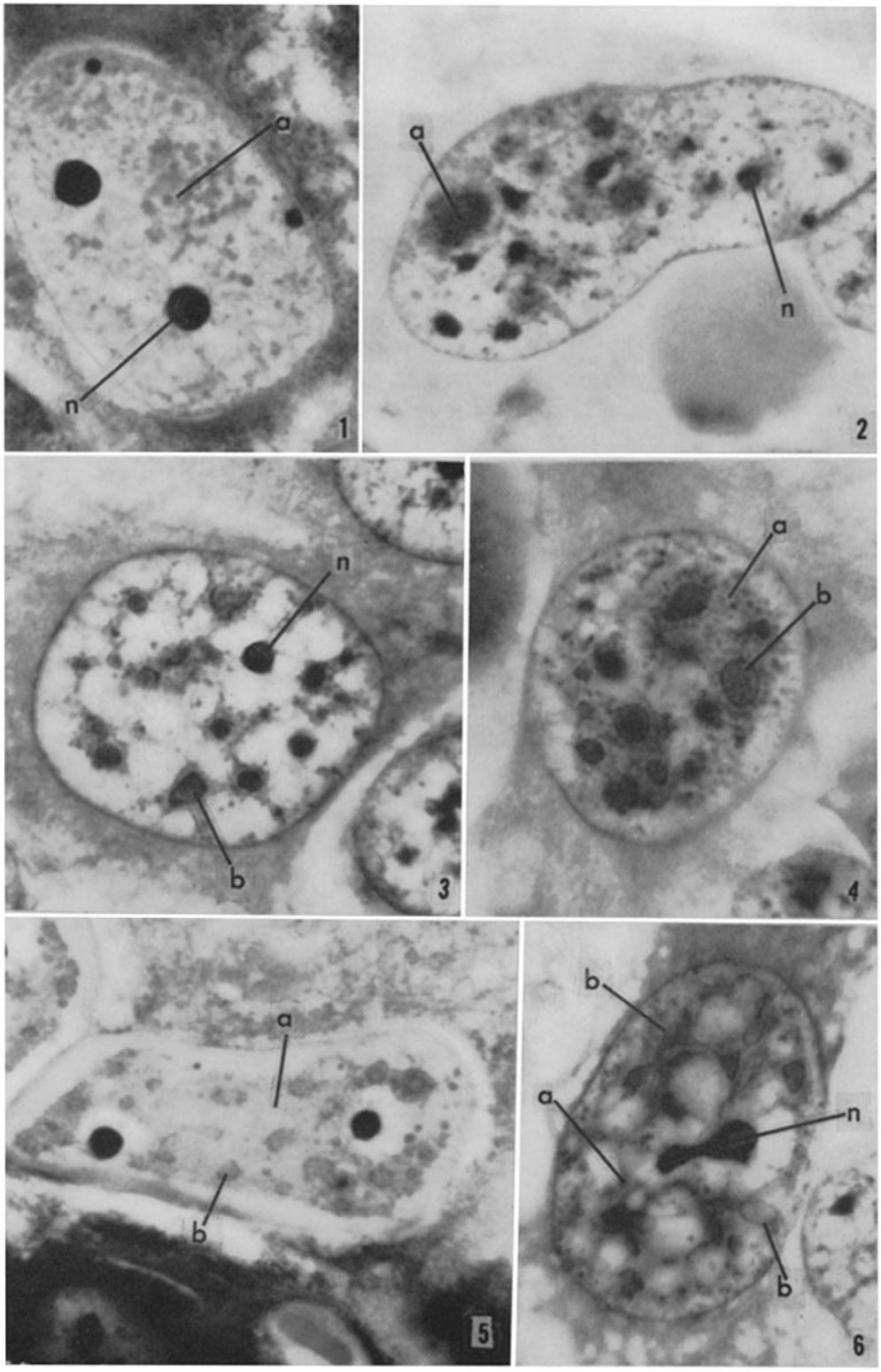

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PLATE 34

FIG. 7. Nucleus of HEp-2 cell, 26 hours after infection, stage 3. The trabecular skein is more compact, and tends to condense toward the center of the nucleus as the central mass (*cm*). The original acidophilic material (*a*) (grey in the photomicrograph) is heavily charged with intensely basophilic granules, masses, and filaments (black in the photomicrograph) which interlace throughout it. The trabeculae enclose locules containing a chromophobic to slightly eosinophilic material, the matrix (*m*), which also occupies the space between the central mass and the distended nuclear membrane. Whole mount, azure-eosin. × 1550.

FIG. 8. Nucleus of HEp-2 cell, 26 hours after infection, late stage 3. The central mass (*cm*) has condensed further, and is represented by an eccentric ring of compacted densely basophilic material enclosing a space or locale containing matrix material (*m*) and a persisting nucleolus (*n*). Matrix material also fills the area between central mass and the nuclear membrane. Whole mount, azure-eosin. × 1550.

FIG. 9. Nucleus of HEp-2 cell, 24 hours after infection, late stage 3. The central mass, consisting of condensed, rather homogeneous eosinophilic material laced with dense basophilic granules, masses and filaments (*g*), encloses two locules. A persistent nucleolus (*n*) lies in one of them; a persistent vesicular body (*b*) lies in its lower pole. Within the matrix material between central mass and nuclear membrane four small asymmetric rectangular bar-like crystals (*c*) are found. Whole mount, azure-eosin. × 1550.

FIG. 10. Nucleus of HEp-2 cell 32 hours after infection, early stage 4. The rosette or flower-like form of the nucleus is conspicuous. The central mass (*cm*) consisting of condensed homogeneous eosinophilic material interlaced with basophilic strands or filaments (*f*), spans the greatly distended nucleus. In this nucleus there is a relatively small amount of basophilic material, and the central mass is only partly condensed. It partly encloses locules of matrix material, bounded externally by the nuclear membrane in which lie several bar-like crystals (*c*). Whole mount, azure-eosin. × 1550.

FIG. 11. Nucleus of HEp-2 cell 24 hours after infection, stage 1. Feulgen-stained material, shown as black in the photomicrograph, is chiefly chromatin; the semicircular or annular formations are nucleolus-associated heterochromatin. The material (*a*) shown as greyish in the photomicrograph is rendered more visible by refractive index difference. It is not stained with the Feulgen reaction, and corresponds to the acidophilic material (*a*) of Figs. 1 and 2. On closer scrutiny, small ring forms probably precursors of the larger vesicular bodies, can be discerned within it. Whole mount, Feulgen reaction. × 1500.

FIG. 12. Nucleus of HEp-2 cell 24 hours after infection, stage 3. The central mass (*cm*) is composed of compacted homogeneous material, not stained in the Feulgen reaction and appearing grey in the photomicrograph, studded with dense Feulgen-stained granules and filaments (*g*). The former material corresponds with (*a*) in Fig. 7, the latter with the basophilic material of Figs. 7 and 9. Whole mount, Feulgen reaction. × 1500.

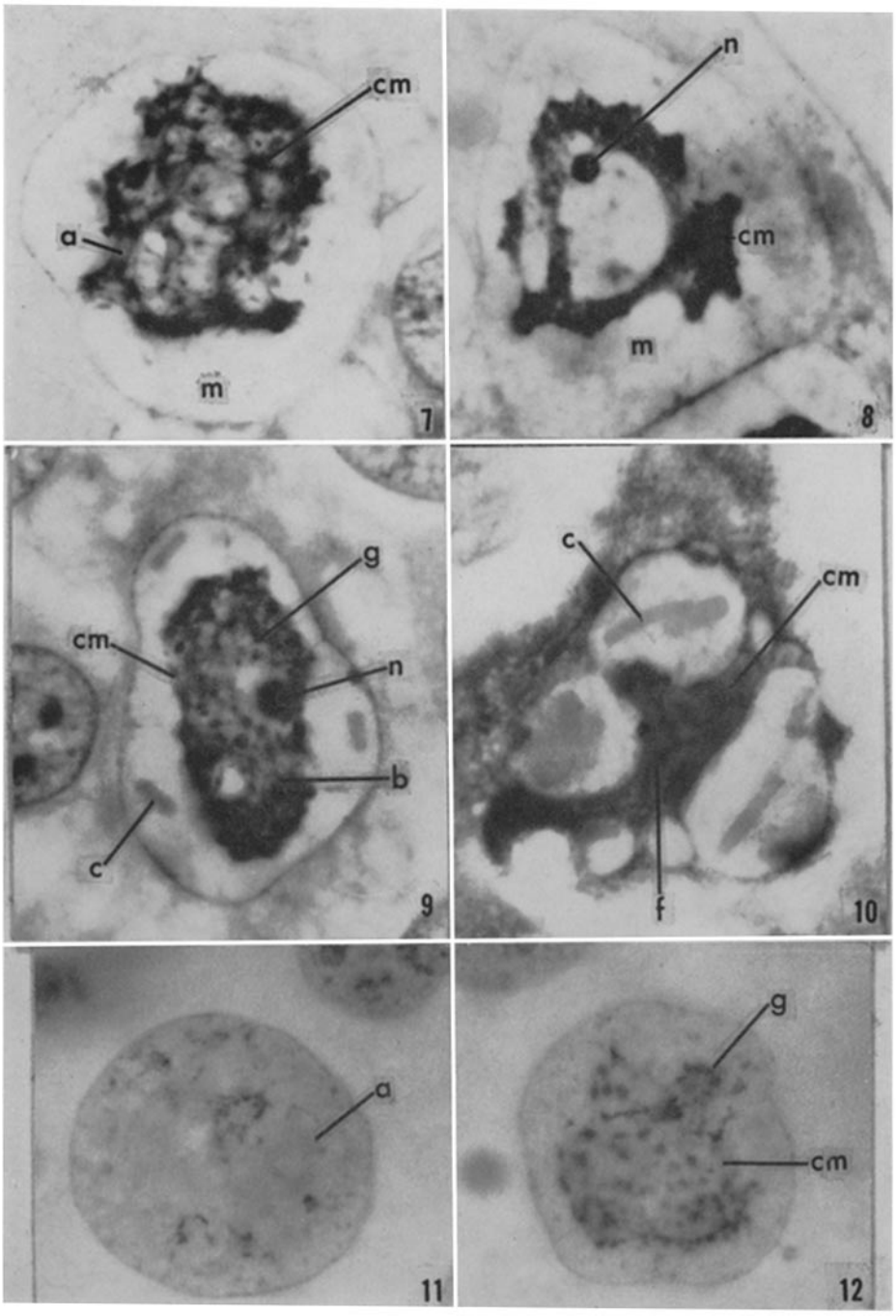

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PLATE 35

All figures except Fig. 18 are of whole mounts stained with azure-eosin.

FIG. 13. Nucleus of HEp-2 cell 36 hours after infection, stage 4. The central mass (*cm*), here disposed into two parts, is extremely condensed and basophilic. Lying in the space between central masses and nuclear membrane is a dense amorphous acidophilic material representing altered, seemingly inspissated or solidified matrix material (*m*). × 1550.

FIG. 14. Nucleus of HEp-2 cell 36 hours after infection, early stage 4. The central mass (*cm*) is not fully condensed, and contains relatively little basophilic material. Bar-like rectangular acidophilic crystals (*c*) lie in each of the locules enclosed by the trabeculae of the mass. × 1550.

FIG. 15. Nucleus of HEp-2 cell 36 hours after infection, stage 4. The rosette or flower-like arrangement is typical. The central mass (*cm*) is extremely compacted and basophilic. The large bar-like crystals (*c*) are arranged around the central mass; the space in which they lie contains a faintly tingible matrix material. × 1550.

FIG. 16. Nucleus of HEp-2 cell, 36 hours after infection, late stage 4. Rosette form similar to that of Fig. 14. Basophilic material is present in the matrix space, chiefly in deposits (*v*) forming heaps at the angles between the acidophilic crystals (*c*) and cuffs or sleeves about them. × 1550.

FIG. 17. Nucleus of HEp-2 cell, 32 hours after infection, stage 4. A rare finding in which the central mass (*cm*), acidophilic bar-like crystal (*c*) and diffuse basophilic material (*v*) typical of stage 5 are present in a poorly circumscribed nucleus with well formed chromosomes (*chr*), as though in pro- or metaphase. × 1300.

FIG. 18. Nucleus of HEp-2 cell 36 hours after infection, late stage 4. The central mass (*cm*) and the more diffuse basophilic material at the angles of the crystals (*c*) are Feulgen-stained. The bar-like acidophilic crystals (*c*) are unstained. Whole mount, Feulgen reaction. × 1950.

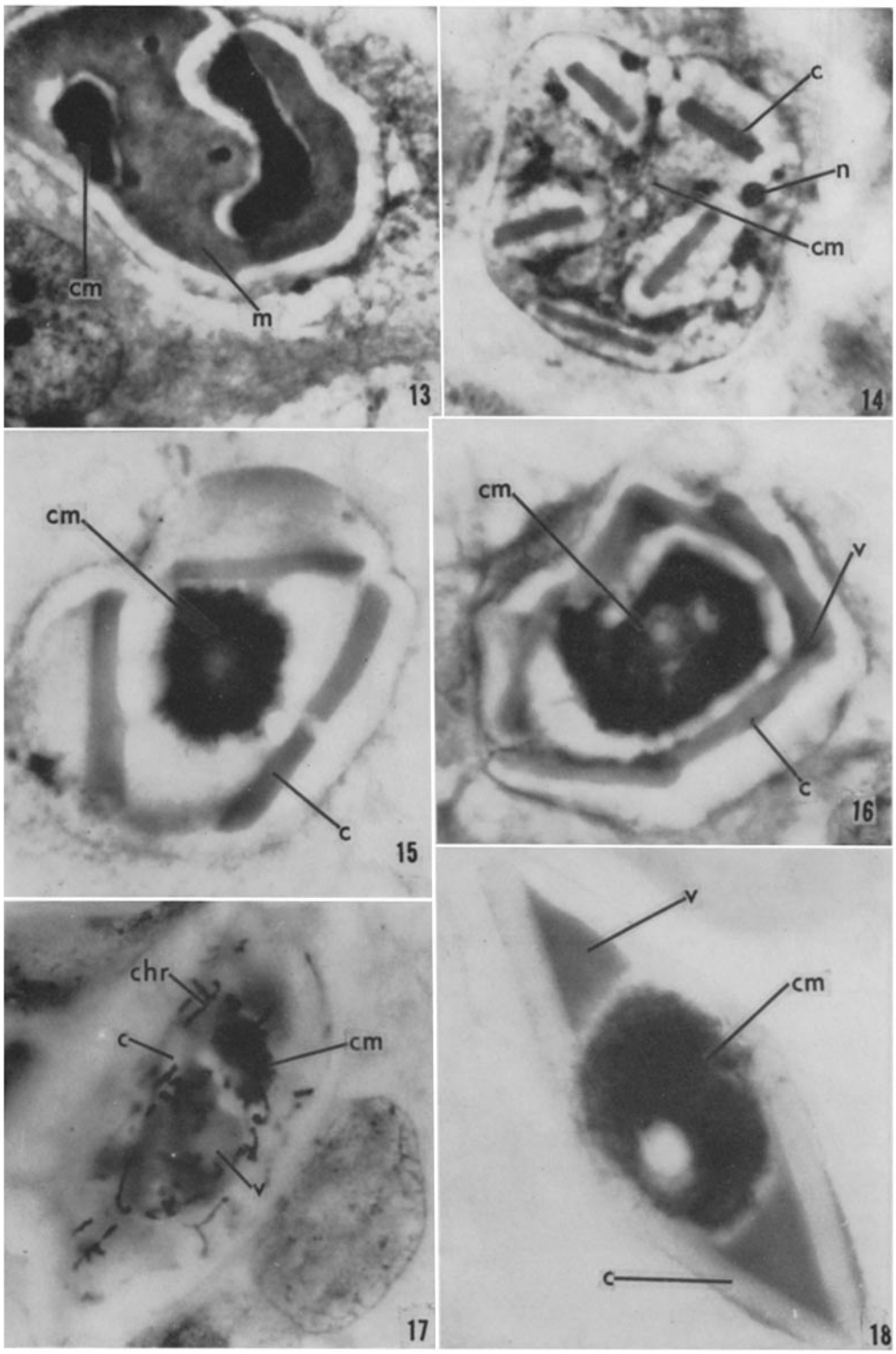

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PLATE 36

FIG. 19. Two HEp-2 cells, 24 hours after infection, stage 1. Masses and floccules of the acidophilic material which will form aggregates and trabecular skeins is shown at (*a*). Several nucleoli are present in each nucleus. Whole mount, azure-eosin. × 850.

FIG. 20. Same cells as those in Fig. 19. Only the nucleolar associated heterochromatin (rings) and some occasional granules are stained with the Feulgen reaction; the acidophilic material is negative. Feulgen reaction. × 850.

FIG. 21. Two HeLa cell nuclei 36 hours after infection, stage 4. The dense basophilic central masses are evident. The matrix material (*m*) lying between central masses and nuclear membranes, here shown as diffuse greyish floccules, is moderately acidophilic, and unusually prominent. Whole mount, azure-eosin. × 1550.

FIG. 22. Same nuclei as those in Fig. 21. The central masses are Feulgen-stained; the matrix material is unstained. Feulgen reaction. × 1550.

FIG. 23. Section of a group of infected cells 36 hours after infection. The crystals (*c*), matrix (*m*) and central masses (*cm*) are all stained in the Millon reaction for tyrosine residues. 5 μ section of pellet, modified Millon reaction. × 500.

FIG. 24. Section of a HEp-2 nucleus containing a bar-like crystal (*c*). The crystal and the material deposited against the nuclear membrane are stained in the coupled tetrazonium reaction for cyclic amino acid residues. 5 μ section of pellet. × 1100.

FIG. 25. Section of a HEp-2 nucleus in stage 4, containing a condensed central mass (*cm*) and a large crystal (*c*). The protein of the central mass is stained for arginine residues; that of the crystal remains unstained. 6 μ section of pellet; modified Sakaguchi reaction for arginine. × 750.

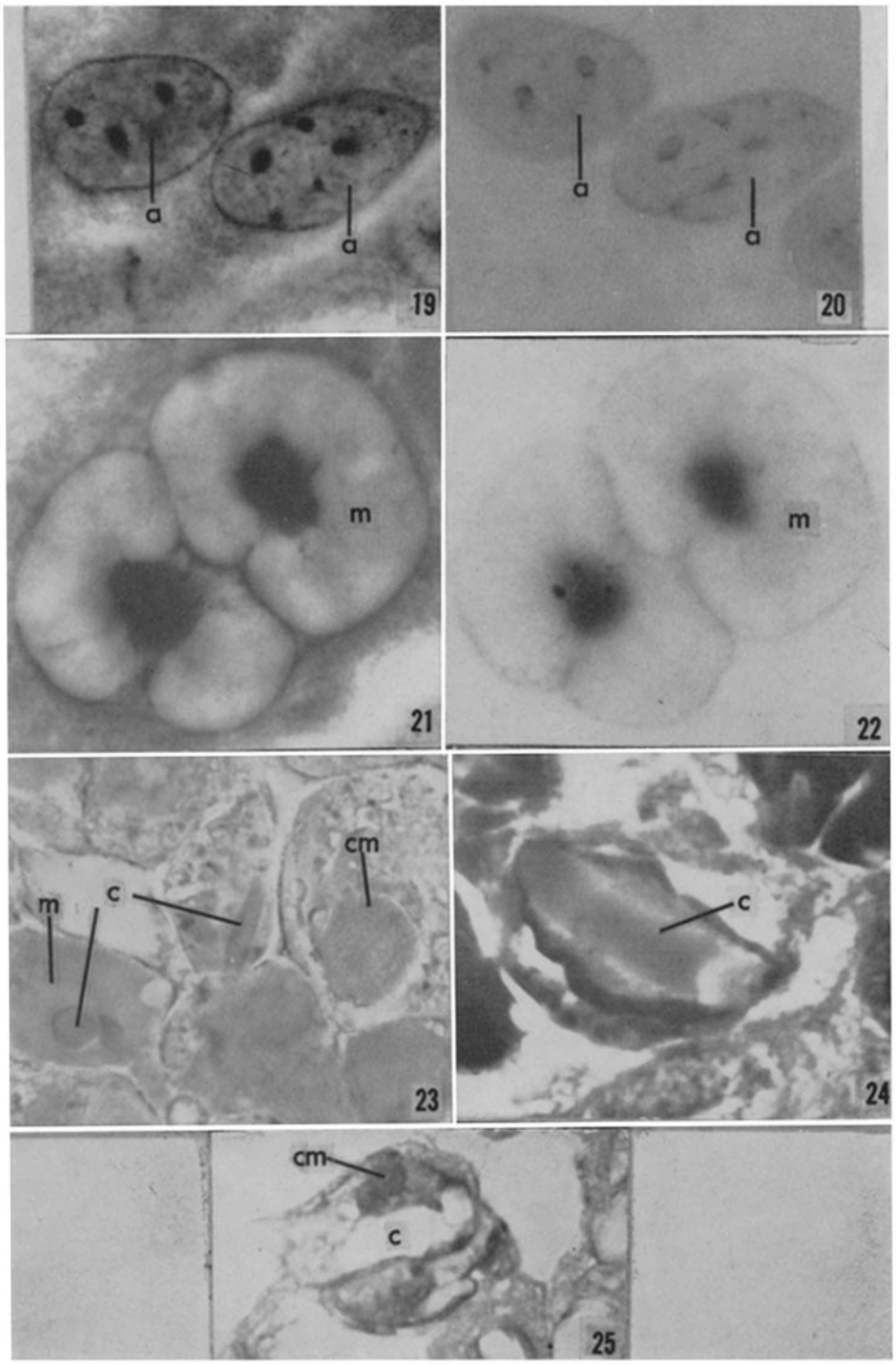

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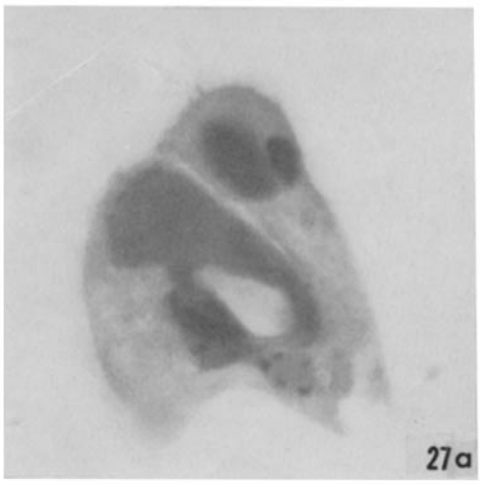
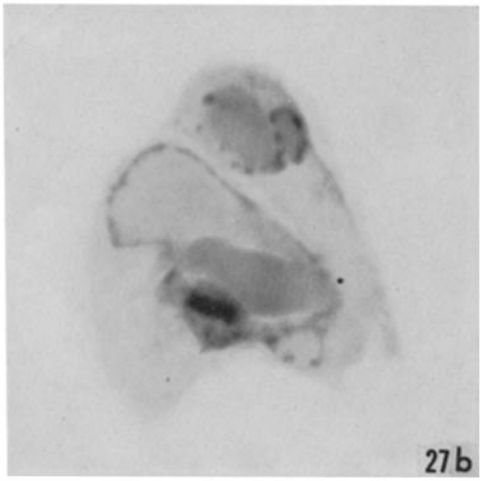
PLATE 37

FIG. 26. Electron micrograph of part of a nucleus of unciform shape containing a large protein crystal. Particles of dispersed virus are present in the nucleus. At the lower left there are clusters of virus and some floccules of the reticular material. × 10,900.

FIG. 27 a. Adjacent thick (2 μ) section of same cell after the Feulgen procedure. The crystal is shown as a negative image which does not stain because it lacks appreciable concentrations of DNA. DNA-containing viral particles and chromatin are colored. × 1,600.

FIG. 27 b. Adjacent thick section of same cell after staining with fast green FCF FCF at pH 2.4. Protein-containing structures including the crystal are stained. × 1,600.





(Godman *et al.*: Development of type 5 adenovirus. II)