A LABILE INTRANUCLEAR RNA ASSOCIATED WITH THE DEVELOPMENT OF ADENOVIRUSES*

By HEATHER DONALD MAYOR, PH.D.

(From the Department of Virology and Epidemiology, Baylor University College of Medicine, Houston)

PLATES 54 TO 57

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The fluorochrome acridine orange (AO) is a sensitive and specific stain for identifying cellular and viral nucleic acids (1-3). Under controlled conditions of dye concentration and pH, acridine orange staining combined with relevant enzyme digestion tests can provide a simple and rapid means for differentiating between not only RNA and DNA but also between the single (S-S) and double stranded (D-S) configurations of DNA (Table I).

The standard staining procedure (4) is in general quite adequate for demonstrating intracellular double stranded DNA and RNA. However it is a common observation that if hydrating and staining solutions are not freshly prepared or the staining procedure is unduly prolonged there is usually a marked deterioration in brilliance particularly of RNA components. In addition, histologists have long been aware that electrolytes in solution can remove variable amounts of RNA from fixed tissues. We were, however, interested in improving sensitivity in order to detect possibly small quantities of labile or unusual nucleic acids arising in the infected cell as a result of viral stimulus. To this end, during the staining procedure, all electrolytes in the staining and hydrating solutions were kept to a minimum and the total processing time made as brief as possible. The specific affinity of the acridine orange dye for the phosphate group of nucleic acid molecules was an additional aid, for if the fluorochrome is introduced immediately after the initial fixation period and retained in each subsequent solution the bound dye molecules should render the nucleic acids less prone to non-specific extraction. Monolayer cultures of normal tissue culture cells treated in this manner stain particularly brilliantly for nucleolar and cytoplasmic RNA. (Figs. 1 and 7).

A sensitive staining procedure for nucleic acids opens the way for a direct cytochemical approach to the mode of information transfer in virus-host cell systems. It is well established that a number of DNA viruses, in particular the papova group (5), can elicit a cytolytic response in one host, and an oncogenic

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one in another, and that a pattern of slow growth in a relatively resistant host is conducive to tumor production. The growth cycle (in tissue culture) of another DNA virus, adenovirus, is marked by a long eclipse period continued cell association of mature virus, and prolonged release in low concentration. It has also been shown recently that human adenoviruses types 12 and 18 can cause tumors in animals (6, 7). The present report focuses attention on the growth cycle in tissue culture of two "cytolytic" adenoviruses (types 3 and 7). It is hoped to extend the studies to other DNA viruses of known oncogenic capacity.

TABLE I

Cytochemical Identification of Viral and Cellular Nucleic Acids

Carnoy-fixed preparation	Tissue culture or virus smear	Color reaction with 0.01 per cent AO at pH 4	Enzyme susceptibility		
			DNAase	RNAase	Pepsin
D-S DNA	Animal virus	Yellow-green (orthochromatic)	+	−	Necessary
D-S DNA	Bacteriophage Cellular chromatin	Yellow-green (orthochromatic)	+	−	−
S-S DNA		Red (metachromatic)	+	−	−
RNA	Virus Nucleolus Ribosomes	Red (metachromatic)	−	+	−

D-S, double stranded.

S-S, single stranded.

Materials and Methods

Tissue Culture and Viruses.—Monolayer tissue cultures of trypsinized rhesus monkey kidney or baboon kidney epithelial cells were grown on 11 × 22 mm coverslips in Leighton tubes using methods and M-H medium previously described (8). Adenovirus type 3 was supplied by Dr. B. Rubin, type 7 was a recent isolation obtained from Dr. M. Benyesh-Melnick.

Growth of Virus at 37°C.—Cultures were inoculated with 0.1 ml of 10^{-1} dilution of the stock viruses (approximately $10^{3.5}$ TCD_{50} tube in each case). At this dilution cytotoxic effects were not observed. After a 2 hour adsorption period the cultures were washed free of virus, and 1 ml of Melnick's M-E medium was added to each tube for incubation. At 4-hour intervals thereafter, 2 cultures were harvested for titration of intracellular virus, 4 for fluorescent antibody staining, and 6 for cytochemistry including nuclease digestion tests. Uninfected control cultures were processed at the same time and in the same manner.

Virus Assay.—The supernatant fluids were discarded, fresh medium added, and the cultures frozen and thawed 4 times and subjected to 30 seconds sonic oscillation at 10 kc to release intracellular virus. Virus titrations were carried out in monkey kidney cells.

Inhibitors of Protein Synthesis.—Puromycin dihydrochloride (Lederle Laboratories Division, Pearl River, New York) and chloramphenicol (Parke, Davis and Company, Detroit) were used.

Acridine Orange (AO) Staining Technique.—The modifications described in the introductory statement were made to the standard staining procedure with AO used in this laboratory (4). After 5 minutes' fixation in Carnoy's fluid (60 per cent ethyl alcohol, 30 per cent chloroform, 10 per cent glacial acetic acid), hydration in alcohol series containing 0.01 per cent AO, and staining for 8 minutes in 0.01 per cent AO at pH 4 in McIlvaine's citric acid and disodium phosphate buffer, coverslips were rinsed briefly in buffer and mounted on standard microscope slides for examination in the fluorescence microscope. Enzyme digestion tests were performed on similar fixed preparations prior to AO staining. All enzyme-tested specimens were brought to pH 4 McIlvaine's buffer before staining. The following enzymes were used: pepsin 0.02[1] per cent in 0.02 M HCl for 10 minutes; RNAase (5 times crystallized[2]) 0.05 per cent at pH 7 in glass-distilled water for 30 minutes; DNAase (1 time crystallized[3]) 0.01 per cent in 0.025 M veronal buffer containing 0.003 M $MgSO_4$ for 30 minutes.

Fluorescent Antibody Staining Technique.—The globulin fractions of antiadenovirus type 3 rabbit antisera (neutralization serum end point titer 1:300) were conjugated with fluorescein isothiocyanate by the standard procedure. Infected preparations were air-dried, fixed in acetone for 10 minutes, and dried again. Coverslips were incubated with 2 drops of the fluorescein-labeled antibody conjugates in a moist chamber at 37°C for 30 minutes, washed in buffer, and examined in the fluorescence microscope. Development of virus-specific fluorescence was completely inhibited by treatment with unconjugated immune sera prior to staining with the labeled conjugates. Uninfected control preparations did not develop any detectable fluorescence when stained with the fluorescent conjugate.

Details of fluorescence microscopy and photomicrography have been described (4).

Electron Microscopy.—Infected, non-infected, and puromycin-treated tissue culture monolayers were fixed *in situ* with 1 per cent cold buffered osmium tetroxide pH 7.2 for 10 minutes, dehydrated rapidly in ethanol series, and impregnated for 2 hours in a mixture of epoxy resin, CIBA 502, and propylene oxide (1:1). The cells were then scraped from the glass surface and embedded as a pellet in fresh resin containing DMP 30[4] as accelerator. Control preparations after cold fixation in 1 per cent buffered osmium tetroxide were treated in the same manner. Ultrathin sections were cut on a si-ro-flex ultramicrotome equipped with glass knives, mounted on carbon-coated copper grids, and examined in the Siemens elmiskop I at magnifications ranging from 5000 to 10,000.

RESULTS

The monkey kidney–adapted strains of adenoviruses used in the study had a latent period of approximately 16 hours after which the titers of intracellular virus rose steeply attaining a maximum value approximately 24 hours post-inoculation (Text-fig. 1). Although the typical cytopathic effect due to adenovirus infection could not be detected until 20 hours after infection, changes in the nucleic acid pattern of infected cells were visible with acridine orange staining as early as 12 hours after inoculation. An enlarged nucleus containing brilliant hypertrophied nucleoli (Figs. 2 and 8) was the first indication of

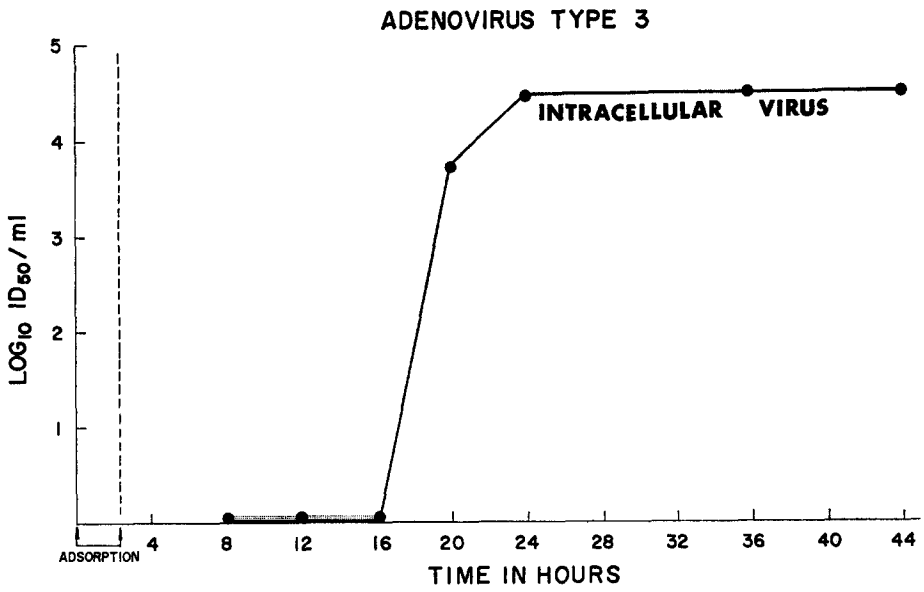
[1] Nutritional Biochemicals Corp., Cleveland.

[2] Pentex, Inc., Kankakee, Illinois.

[3] Union Carbide Chemicals Co., New York.

[4] 2,4,6-Tri(dimethylaminoethyl)phenol, Rohm and Haas Co., Philadelphia.

infection, followed by the development of a green delimited inclusion of DNA within the nucleus (Figs. 2 and 9). This early inclusion was readily susceptible to digestion with DNAse without proteolytic enzyme treatment. 20 hours after inoculation small bright dense particles of yellow-green staining material appeared within the green inclusion, and often a bright ring of an extremely labile RNA surrounded the complete inclusion (Figs. 3, 4, 10, and 11). The cytoplasm of infected cells was usually stained a brilliant red at this stage, and diffuse but specific viral antigen could be detected in the nucleus (Fig. 17).



TEXT-FIG. 1. Growth cycle of adenovirus type 3 in monkey kidney tissue culture.

24 hours after infection the intranuclear inclusion had taken on a more crystalline appearance and was susceptible to DNAse digestion only after proteolytic enzyme treatment, indicating that virus maturation was now complete. In many cells RNA-staining material appeared not only as a ring surrounding the viral inclusion but also ramifying its crystalline matrix (Figs. 5 and 12). Granular and crystalline points of viral antigen and many large brilliant aggregates were detectable at this time (Fig. 18). By 36 hours or later, the nucleoli were no longer evident and brilliant yellow-green inclusions of mature viral DNA completely filled the nuclei (Figs. 6 and 13). No nuclear RNA staining was evident at this time, and the infected cells were rounded up and exhibited the typical cytopathic effect in the light microscope.

Table II taken from a series of experiments performed in our laboratory

with a number of viruses and virus-cell preparations compares the solubility pattern of the labile RNA with cellular and extracellular fixed nucleic acids.

We note in particular the reactions of S-S DNA (as demonstrated by fixed preparations of bacteriophage ΦX174) and labile RNA to dilute alkali. Cytochemical demonstration of labile RNA was completely prevented by briefly dipping the fixed slide in 0.03 per cent NaOH whereas a 10 minute soaking did not dissolve out the S-S DNA. Both ribosomal and nucleolar RNA stained faintly after brief dipping in mild alkali, but in general they required additional brief treatment with RNAse for complete removal. Nuclear chromatin (D-S DNA) and ΦX174 (S-S DNA) readily lost staining brilliance after 30 minutes at 37°C in 2 M NaCl while ribosomal and mature viral RNA (TMV and poliovirus) scarcely suffered any diminution in brilliance. The nuclear RNA in our fixed

TABLE II

Cytochemical Demonstration of Nucleic Acid Solubility

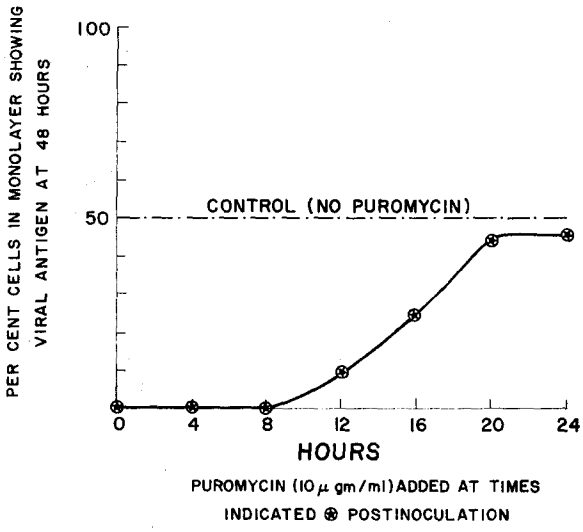
Carnoy-fixed preparation	NaOH (0.03 per cent)	NaCl (0.15 M)	NaCl (2 M)
D-S DNA	Insoluble	Insoluble	Soluble
S-S DNA	Insoluble	Soluble	Soluble
RNA	Soluble	Soluble	Insoluble
Labile RNA	Soluble	Soluble	Soluble

D-S, double stranded.
S-S, single stranded.

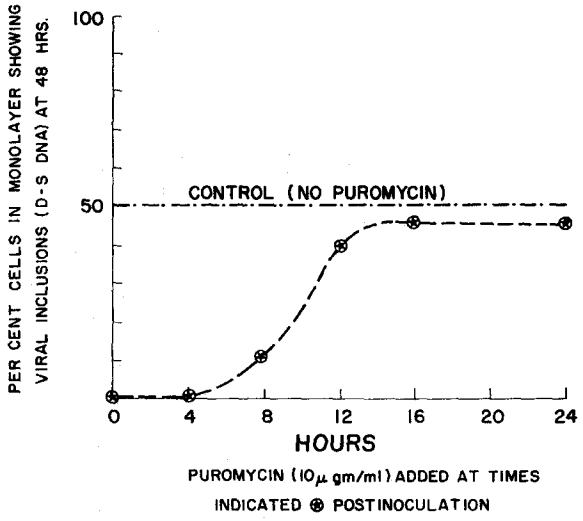
adenovirus-infected cultures was readily and *immediately* removed in both dilute alkali, 0.15 per cent and 2 M sodium chloride.

Additional cytochemical information concerning the basic mechanisms whereby the cell is preempted for viral synthesis can be obtained from studies of this adenovirus system when puromycin, a potent inhibitor of protein synthesis, is added during the replication cycle. When adenovirus is inoculated into cultures which have been maintained in a minimal medium (a balanced salt solution containing adequate glucose) we would expect the nucleic acid and protein synthesizing-mechanisms of the infected cells to be directed almost entirely toward viral replication. Addition of puromycin, at a level of about 10 μg/ml, at the beginning of the latent period, resulted in an almost complete inhibition of production of antigenic protein as detected by fluorescein-labeled antibodies (Text-fig. 2). However, development of the typical double stranded DNA viral inclusion continued for a time except when puromycin was added within the first hours after infection (Text-fig. 3). Most of this DNA was susceptible to digestion by DNAse without pepsin treatment indicating that virus maturation has not taken place.

When the drug was added towards the end of the latent period, *i.e.* about



TEXT-FIG. 2. Growth of adenovirus type 7 in presence of puromycin as revealed by the number of cells in the monolayers showing inclusions containing viral antigen 48 hours after nfection. In control preparations 50 per cent of the cells showed inclusions at this time.



TEXT-FIG. 3. Growth of adenovirus type 7 in presence of puromycin as revealed by the number of cells in the monolayers showing inclusions of viral DNA 48 hours after infection. In control preparations 50 per cent of the cells showed inclusions at this time.

16 hours after inoculation, when labile RNA could be readily detected in infected control cells, additional RNA was accumulated in the nucleus of some cells and could be demonstrated cytochemically (Figs. 14–16). Observations were made in preliminary experiments using chloramphenicol as a protein

inhibitor. However complete inhibition of viral protein was not obtained unless levels approaching 100 μg/ml of the drug were employed. These large doses were markedly toxic to the cell monolayers and seriously interfered with interpreting the results. Use of chloramphenicol was therefore discontinued.

When replicate cultures were examined in the electron microscope in ultrathin sectioned preparations a marked margination of nuclear chromatin was observed in presence of puromycin (Fig. 21). There was also increased activity of the microvilli at the plasma membrane (Fig. 22). In preparations where puromycin was added at time zero or up to 8 hours after inoculation dense particles approximately 25 to 30 mμ in diameter could be detected in the nucleus of infected cells (Fig. 19), but there was little evidence of viral capsid formation. In control-infected preparations and those where puromycin was introduced later in the replication cycle (16 hours after incubation) there was marked evidence of formation of coat protein and many complete virus particles approximately 65 mμ in diameter were found (Fig. 20). It would appear that puromycin inhibits the development of capsid protein but that synthesis of viral DNA can continue, at least for a time.

DISCUSSION

In tissue culture cells infected with the double stranded DNA viruses, adenoviruses type 3 and 7, technical modifications of the standard cytochemical procedure with acridine orange to detect nucleic acids have led to the observation of a labile nuclear RNA distinct from nucleolar RNA appearing from 16 to 20 hours after inoculation. This is shortly before assay methods demonstrate the formation of mature virus particles and before the typical viral inclusion consisting of protein and double stranded DNA can be observed in the nuclei by cytochemical and immunofluorescent procedures (Figs. 13 and 18). We have no direct experimental evidence that this RNA indeed carries the message for assembling specific viral protein, but its time of appearance and location surrounding and ramifying the viral inclusion together with its extreme lability and rapid disappearance as soon as the mature viral inclusion is fully formed are highly suggestive that it is informational RNA involved in the formation of adenovirus protein. We have not been able to detect a similar RNA in the nuclei of uninfected cells or cells infected with a RNA virus (poliovirus) where virus protein is obviously fabricated in the cytoplasm. It is clear that we can detect this labile RNA for a limited time only.

This RNA has novel properties and is unlike any RNA previously demonstrated by acridine orange staining. How do we know that it is indeed RNA? It stains red and is immediately susceptible to RNAse and untouched by brief treatment with DNAse. Its cytochemical behavior marks it as an extremely soluble RNA with the following properties.

1. Its appearance in the cell is brief and transient, indicating that it is being rapidly utilized in the infected cells.

2. It is difficult to detect and has only been demonstrated using the improved staining procedures described here.

3. It is extremely labile, and its demonstration is *invariably* the first property to be lost with the mildest extraction procedures.

Our experiments with puromycin-treated cells indicate that under certain conditions quite large amounts of soluble nuclear RNA can be observed. This result would appear to indicate that the RNA is prevented from expressing its true function by the presence of patent protein inhibitors and that during the normal cycle of viral infection this nuclear RNA is involved in synthesis of adenovirus protein. In a recent review (9) von Bertalanffy has speculated that transfer RNAs of quite low molecular weights should be readily detectable by cytochemical techniques.

A possible extension of these experiments could have interesting implications at the molecular level of viral replication for both cytolytic and oncogenic viruses, as the manner in which both RNA and DNA viruses code their specific proteins can be studied by this direct cytochemical procedure.

Recently Pollard and Starr (2) have elucidated a unique biochemical sequence of events coincident with the replication of psittacosis virus in tissue culture using acridine orange staining. The role of a novel cytoplasmic RNA in the maturation of this DNA agent was determined. On the basis of their results and those reported here we can predict an increasing application of fluorochrome procedures in integrated studies on the biochemical mechanisms of viral replication.

SUMMARY

The nucleic acids produced intracellularly during the replication cycles of both DNA and RNA viruses can now be identified rapidly using a sensitized procedure based on staining with the fluorochrome acridine orange. Cellular DNA, viral DNA (both single and double stranded forms), cellular RNA, and RNA arising as a result of viral stimulus can be differentiated.

The intracellular development of virus specific DNA, RNA, and protein has been studied in monkey kidney cells infected with adenoviruses types 3 and 7. It has been possible to detect a labile RNA in the nucleus from 16 to 20 hours after inoculation. When the cultures are treated with puromycin at this time, this RNA can be accumulated under certain conditions in the nucleus and demonstrated cytochemically. At the same time the production of specific viral protein as determined by staining with fluorescein-labeled antibodies is markedly inhibited. However, intranuclear double stranded DNA continues to be formed for a time. When puromycin is added to the system early in the eclipse period virus-specific DNA and labile RNA cannot be detected.

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EXPLANATION OF PLATES

PLATE 54

FIG. 1. Diagram of the nucleus of a normal monkey kidney cell. Two nucleoli (*Nc*) are shown. Nuclear chromatin (*C*) is evenly distributed. Nuclear membrane (*NM*) is clearly delineated.

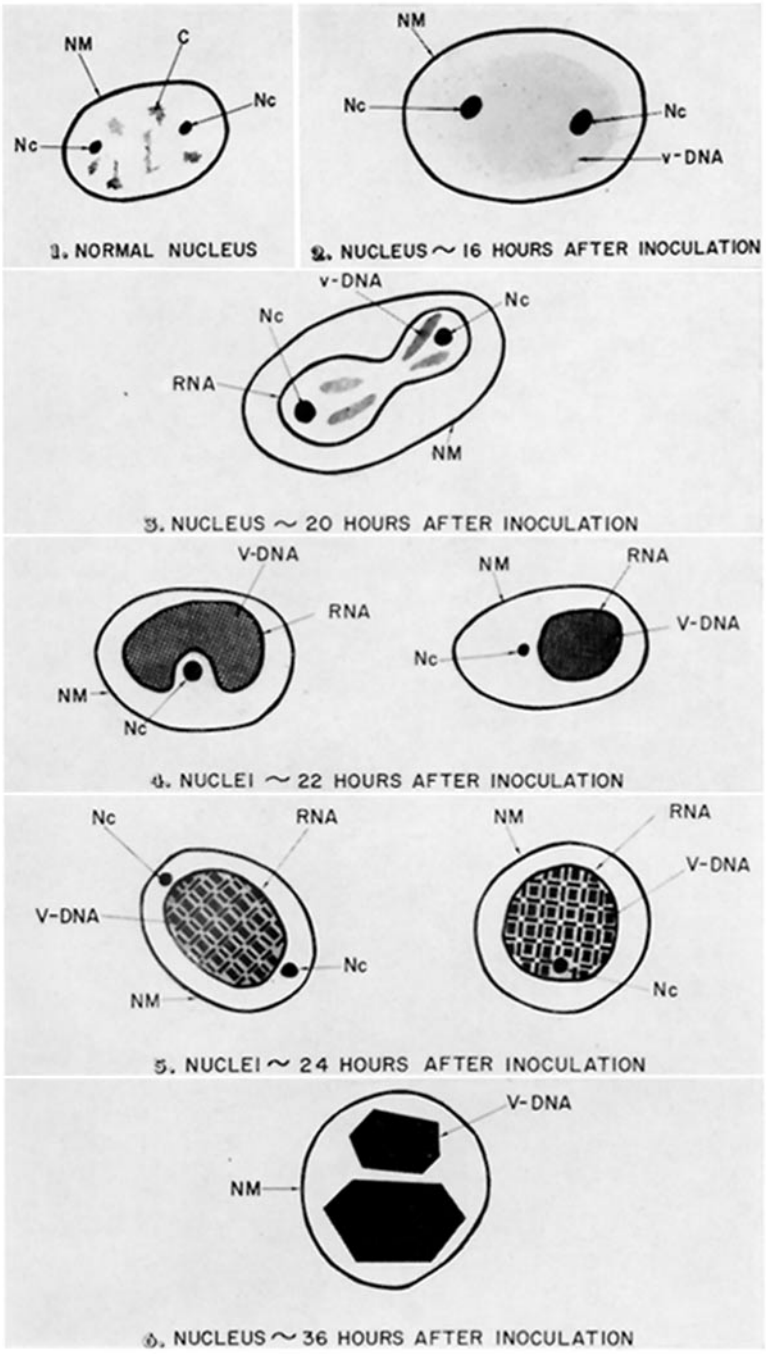
FIG. 2. Diagram of a typical nucleus 16 hours after inoculation with adenovirus. Inclusion of DNAase-susceptible viral DNA (*V-DNA*) is present. Nucleoli (*Nc*) are prominent.

FIG. 3. Diagram of a typical nucleus 20 hours after inoculation with adenovirus. Enormous nucleoli (*Nc*) are present. A bright ring of labile RNA surrounds the viral inclusion.

FIG. 4. Diagram of two typical nuclei 22 hours after inoculation with adenovirus. There is a mature viral inclusion (*V-DNA*), crystalline in nature and susceptible to DNAase only after proteolytic enzyme treatment. Labile RNA surrounds the inclusion.

FIG. 5. Diagram of two typical nuclei 24 hours after inoculation with adenovirus. Labile RNA surrounds and ramifies the mature inclusion of viral DNA (*V-DNA*). Nucleoli (*Nc*) are still evident.

FIG. 6. Diagram of a typical nucleus 36 hours after inoculation with adenovirus. Two mature inclusions (*V-DNA*) are shown. Nuclear RNA and nucleoli are no longer evident.



(Mayor: Labile intranuclear RNA)

PLATE 55

FIG. 7. Normal monkey kidney cells stained with acridine orange. Cytoplasm stains red (RNA) nuclei yellow green (DNA). Nucleoli (black arrows) display a faint pink tinge. × 1250.

FIG. 8. Monkey kidney cells 16 hours after infection with adenovirus type 3. Acridine orange technique. Nuclei are enlarged chromatin clumped. Nucleoli (black arrows) are enlarged and stain brilliantly for RNA. × 1250.

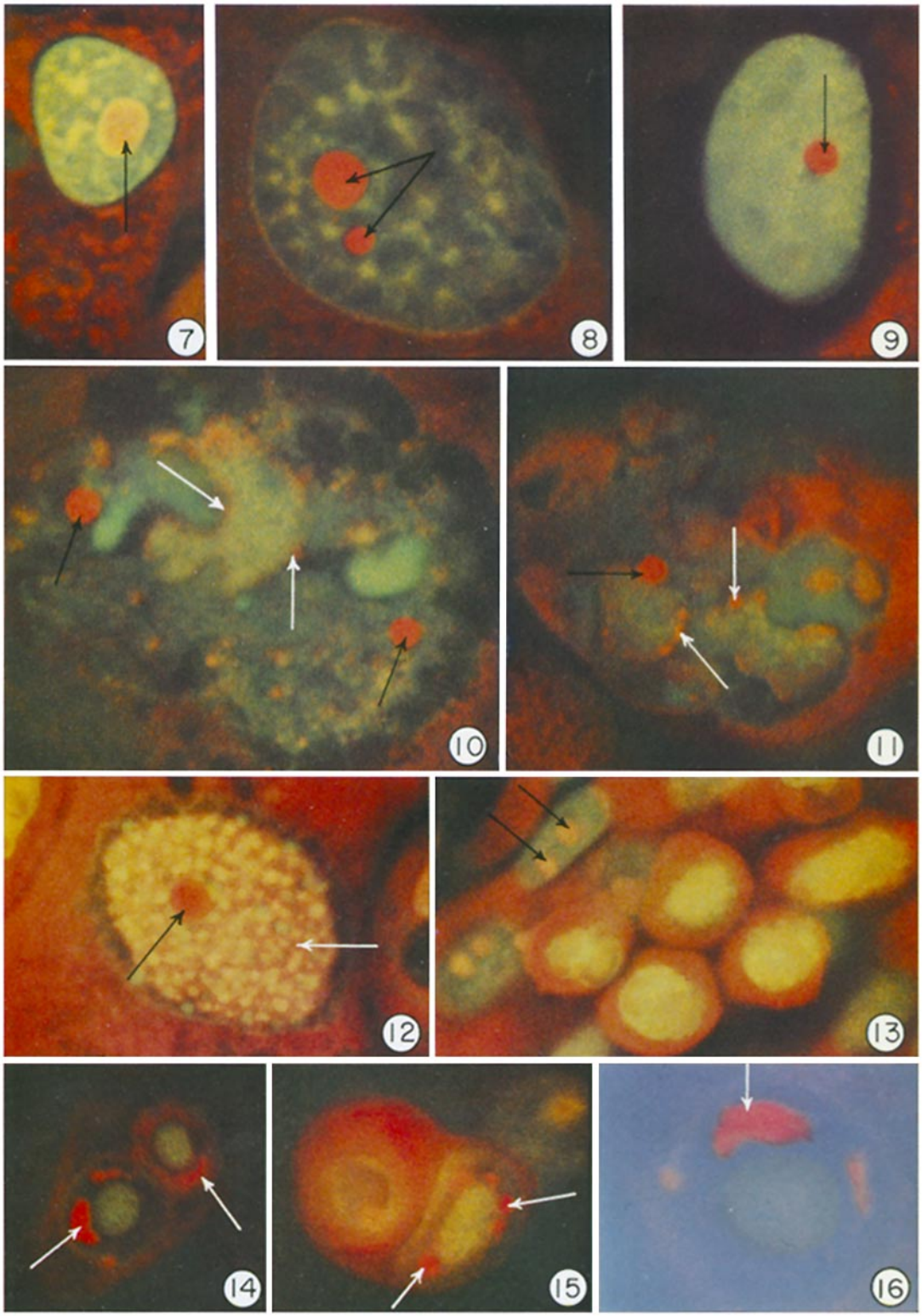
FIG. 9. Monkey kidney cells 16 hours after infection with adenovirus, type 3. Acridine orange technique. Green inclusions of DNA fill the infected nucleus. Staining reaction is susceptible to DNAase without proteolytic enzyme treatment. Nucleolus (black arrow) stains a brilliant red. × 1250.

FIGS. 10 and 11. Monkey kidney cells 20 hours after infection with adenovirus, type 3. Acridine orange technique. Nucleus shown is greatly enlarged. Nucleoli (black arrows) brilliant red. Nuclear DNA inclusion delimited by green fluorescence contains bright patches and is for the most part susceptible to DNAase without proteolytic enzyme treatment. Labile RNA (white arrows) forms a ring surrounding the viral inclusion (see Fig. 3 for its location). × 3000.

FIG. 12. Monkey kidney cells 24 hours after infection with adenovirus, type 3. Acridine orange technique. Nucleoli are a brilliant red (black arrow). Viral inclusion is brilliant and yellow-green, susceptible to DNAase only after proteolytic enzyme treatment. Labile RNA (white arrow) surrounds the viral inclusion and ramifies the crystalline matrix (see Fig. 5). × 1500.

FIG. 13. Monkey kidney cells 36 hours after infection with adenovirus, type 3 acridine orange technique. Nucleoli (black arrows) are no longer evident in heavily infected cells. Brilliant yellow-green crystals of mature viral DNA fill the nuclei. No nuclear RNA is evident. × 1250.

FIGS. 14 to 16. Starved monkey kidney cells 48 hours after infection with adenovirus, type 3. Acridine orange technique. Puromycin (10 μg/ml) has been added 16 hours after inoculation. A green nuclear inclusion of DNA is evident. Brilliant nuclear RNA (white arrows) is concentrated around the inclusion. Fig. 14, × 1500; Figs. 15 and 16, × 1250.



(Mayor: Labile intranuclear RNA)

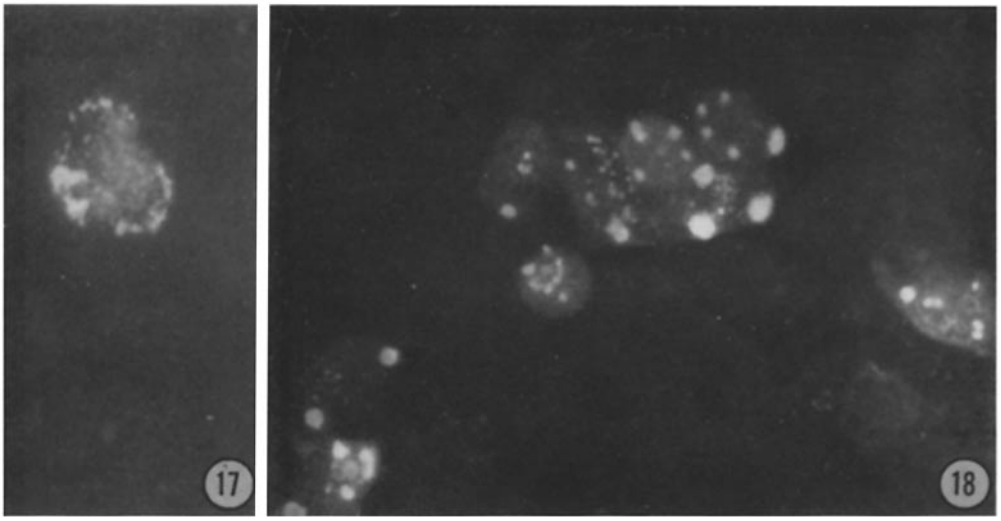
PLATE 56

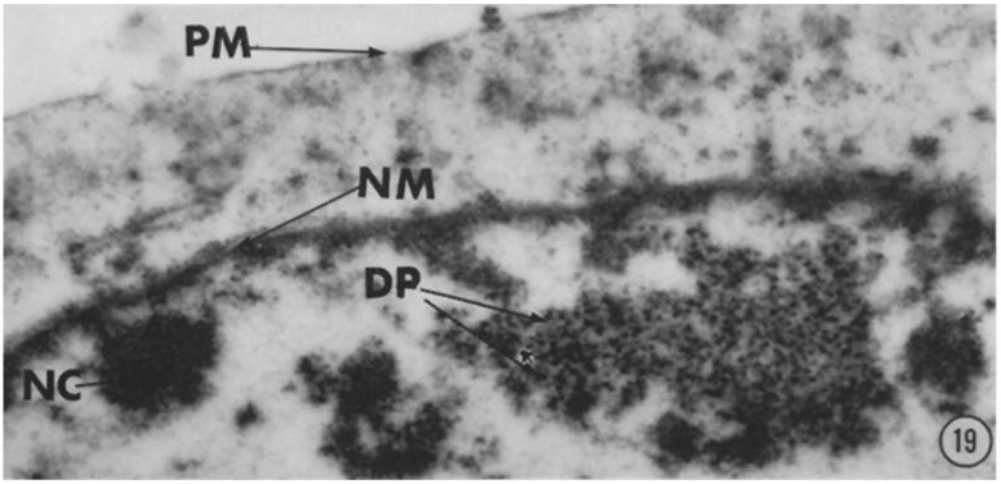
FIG. 17. Nucleus of infected cell 16 hours after inoculation. Fluorescent antibody technique. Diffuse and granular viral antigen is present. × 1000.

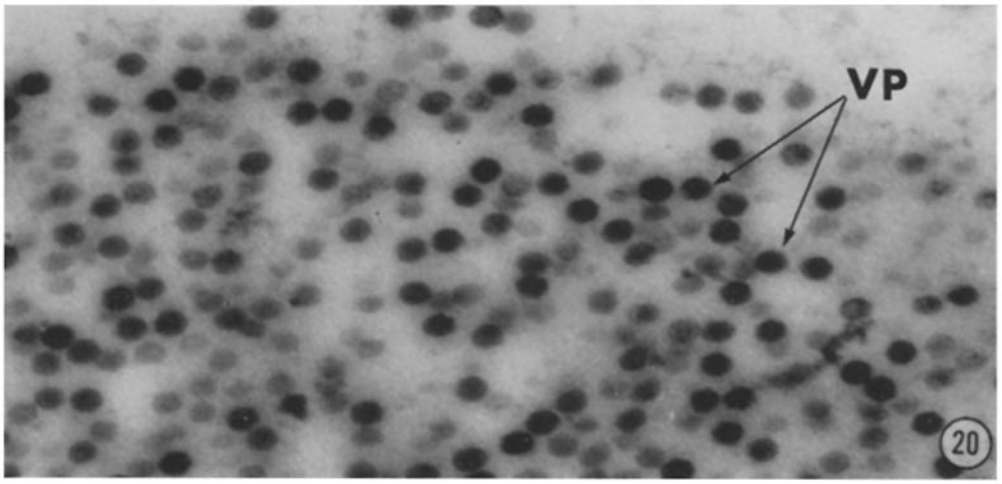
FIG. 18. A group of infected cells 24 hours after inoculation. Fluorescent antibody technique. Brilliant aggregates of viral antigen are present in the nuclei. × 1000.

FIG. 19. Electron micrograph of an ultrathin section of a cell infected with adenovirus type 7. 10 μg/ml puromycin was added 8 hours after inoculation and the culture harvested 48 hours after inoculation. Margination of nuclear chromatin (*NC*) has occurred. Many dense particles (*DP*) are seen often in association with the nuclear chromatin. *NM*, nuclear membrane; *PM*, plasma membrane. × 40,000.

FIG. 20. Electron micrograph of an ultrathin section of a cell nucleus infected with adenovirus type 7. 10 μg/ml puromycin was added 16 hours after inoculation and the culture harvested 48 hours after inoculation. Many complete virus particles (*VP*) are seen. × 60,000.





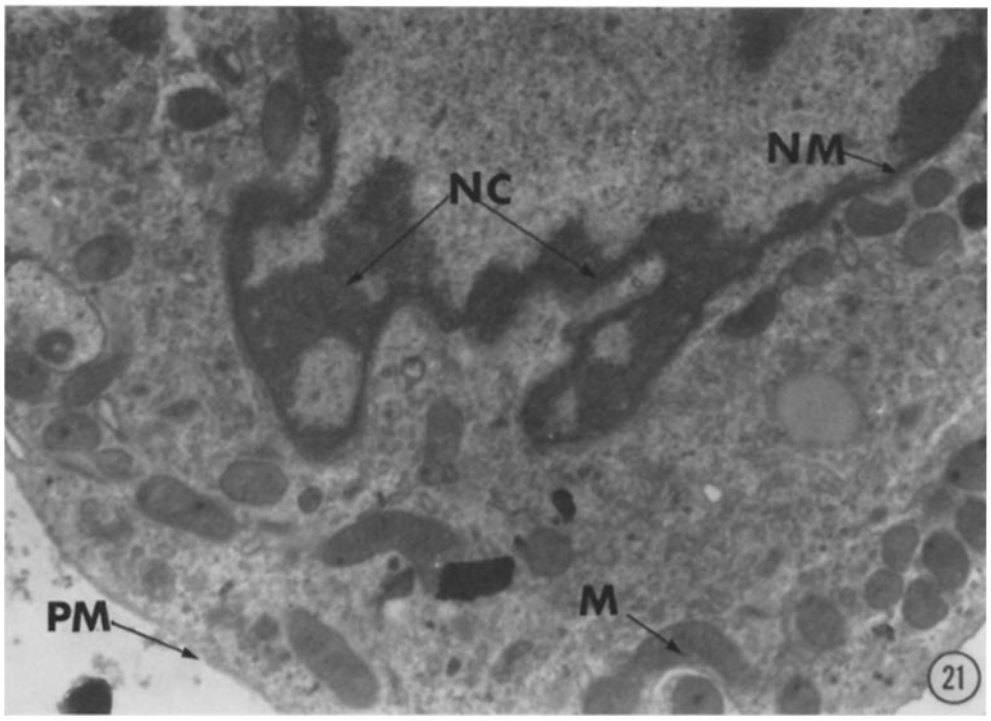


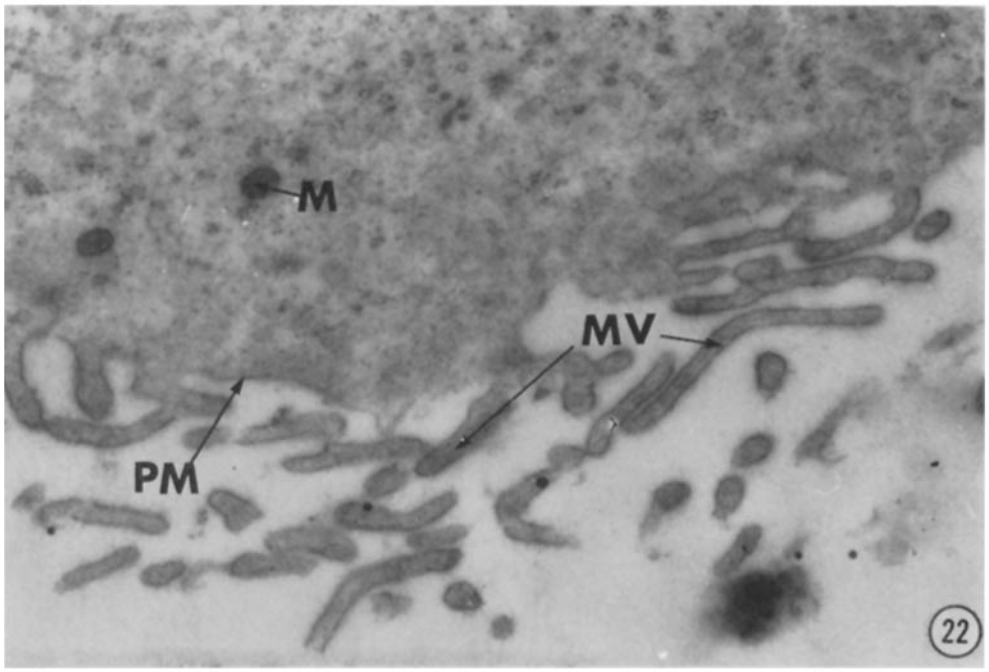
(Mayor: Labile intranuclear RNA)

PLATE 57

FIG. 21. Electron micrograph of an ultrathin section through a monkey kidney cell maintained in the presence of 10 μg puromycin/ml for 24 hours. Marked margination of nuclear chromatin (*NC*) has occurred. *NM*, nuclear membrane; *PM*, plasma membrane; M, mitochondrion. × 30,000.

FIG. 22. Electron micrograph of an ultrathin section through a monkey kidney cell maintained in the presence of puromycin. Many microvilli (*MV*) are visible at the plasma membrane (*PM*). M mitochondrion. × 48,000.





(Mayor: Labile intranuclear RNA)