A CYTOCHEMICAL DESCRIPTION OF THE
MULTIPLICATION OF MENGOVIRUS IN L-929 CELLS

RICHARD M. FRANKLIN, Ph.D.
From The Rockefeller Institute

ABSTRACT
A correlation of cytochemical changes with virus production has been studied in L cells infected with Mengovirus. After a latent period of about 2 hours, virus was produced rapidly, reaching maximum titers of up to 12,000 particles per cell in 6 to 8 hours. The earliest cytological change was in the nucleus and consisted of a slight condensation of chromatin. There is no evidence, however, for the multiplication of either the viral RNA or protein in the nucleus. RNA, of high molecular weight, accumulated in the perinuclear area of the cytoplasm and was later found in inclusions. The perinuclear RNA was digestible with RNase and may be located in or on ribosomes. The inclusion RNA was resistant to RNase but could be removed by pepsin or potassium permanganate; it is probably in completed virus particles.

Viral antigen was first observed in a perinuclear location and later in the above-mentioned inclusions. Although the viral protein contains appreciable amounts of arginine and lysine, it is not a basic protein of the histone type. Phase-contrast microscopy of living cells clearly demonstrated the role of the inclusions in release of virus from infected cells.

A comparison is made between these cytological changes in Mengo-infected cells and those which have been found by other workers in polio-infected cells. There are many very similar changes.

INTRODUCTION
This is one in a series of reports on the multiplication of Mengovirus in L cells. A detailed study of this small RNA virus may provide some clues to the mechanism whereby an animal virus controls synthetic processes within a cell. This is an especially intriguing problem in the case of RNA animal viruses since synthesis of many such viruses does not seem to depend on the genetic apparatus of the cell (1-3). Neither host cell DNA synthesis (1), nor the presence of any particular host cell genome (2), nor the continued synthesis of normal host cell RNA (3) is required for the synthesis of many RNA animal viruses, including Mengovirus. These facts have stimulated interest in the site of Mengovirus multiplication within the cell. The present work is concerned with cytochemical changes in the cell during the course of virus synthesis. While the cytochemical approach cannot be expected to be complete in itself, it can indicate some of the events occurring during virus multiplication and thereby suggest further approaches to the problem.

Mengovirus belongs to the Columbia SK group of closely related mouse encephalomyelitic viruses (4, 5). These viruses are approximately 25 to 30 mμ in diameter and probably contain only RNA and protein. All members of the Columbia SK group are antigenically related, indeed may be antigenically identical (6). The mouse encephalomyelitis virus recently studied by the present author (7) also belongs to this group of viruses (8).
MATERIALS AND METHODS

1. Biological Techniques

The origin of the virus used in these studies, routine cell techniques, and biological assays have been described elsewhere (9). The original stock of Mengovirus contained a mixture of plaque-size variants. A small plaque variant was purified by three consecutive plaque isolations and used in these studies as a source of genetically pure virus. Unfortunately, this particular strain mutated to a larger plaque variant with rather high frequency so that it has been difficult to maintain the small plaque variant. Both large and small plaque variants seemed, however, to produce the same cytochemical effects.

For virus growth studies, cells were grown on plastic Petri plates, 60 mm in diameter (Falcon Plastic Co., Los Angeles, Calif.). For cytochemical studies, cells were grown on No. 1 coverglasses, 22 mm sq. Infection of cells, either on plastic plates or on coverslips in plastic plates, was initiated with 0.4 ml of an appropriate dilution of stock virus which was adsorbed to the cells by incubation for 30 minutes at 37°C in 5 per cent CO2/95 per cent air. The virus inoculum was redistributed on the plates every 10 minutes by tilting. The inoculum was removed and the plates rinsed with Eagle's minimum essential medium (MEM), twice for cytochemical studies and four times for virus growth studies. The nutrient medium for all experiments consisted of MEM with 5 per cent selected horse serum (9). In virus growth studies, total virus was collected by harvesting cells and supernate together, freezing and thawing three times to disrupt the cells, and removing cell debris by centrifugation (1000 rpm 10 minutes). Released virus was assayed by sampling the nutrient medium.

Both in cytochemical and virus growth experiments a virus inoculum sufficient to infect all cells was used. An estimate of the multiplicity of infection can be made as follows. In cytochemical experiments relatively few cells were plated so that they would be well separated and easy to study. The virus inoculum, however, is titrated on a monolayer of cells. When added to a plate of fewer cells, it is still distributed over the entire surface, irrespective of the presence or absence of cells. The average area of well spread L-929 cells was measured from phase-contrast micrographs of living cells and found to be 450 \( \mu^2 \) or 4.5 \( \times 10^{-4} \) cm\(^2\). Therefore, a perfect monolayer of cells on a round Petri plate, 6 cm in diameter, would consist of approximately 6 \( \times 10^9 \) cells.

In the experiments reported here, this area is exposed to 4 \( \times 10^6 \) plaque-forming units (PFU) of virus in a volume of 0.4 ml for 30 minutes at 37°C. Under these conditions, maximum amounts of virus have been adsorbed by 30 minutes (9). This is a multiplicity of 6.7 PFU/cell, which means that 100

\[ (1 - e^{-6.7}) \] per cent = 99.9 per cent of the cells are infected after a 30-minute adsorption period. These estimates are supported by the cytochemical studies which show synchronized changes in most of the cells during a single cycle of virus multiplication.

Hemagglutination assays were done using sheep red blood cells, with modifications of the procedure of Hallauer (10). 0.5 ml of a 0.15 per cent suspension of red blood cells in 4.5 per cent glucose with 0.085 per cent NaCl was mixed with 0.5 ml of diluted virus in 0.85 per cent NaCl. Hemagglutination was read after incubation for 18 to 24 hours at 4°C.

Rat convalescent serum was used as antiserum to Mengovirus. A single intraperitoneal dose of 10° PFU was given and the surviving rats were bled after 30 days. These sera had hemagglutination inhibition titers up to 1:10,000.

2. Cytochemical Techniques

Several procedures were used for fixation, depending on the stains to be employed. The various combinations of fixatives and stains will be mentioned in the results.

b) Fixation Procedures

A. ACETIC ALCOHOL: Absolute ethanol/glacial acetic acid (3:1) was prepared daily and chilled at -30°C. Cells were rinsed in phosphate buffer, dipped into acetic alcohol at 4°C for 5 seconds, and then fixed in a second change of acetic alcohol at 4°C for 10 minutes. This was followed by three washes in 70 per cent ethanol at 4°C. Cells could be stored several days in 70 per cent ethanol at 4°C or brought to water and stained immediately.

B. FREEZE-SUBSTITUTION: Cells were rinsed in phosphate buffer, quenched in absolute ethanol/dry ice, and substituted in absolute ethanol/methanol (1:1) at 30°C for 60 minutes, then dried rapidly in a stream of cold air at 4°C. In some cases cells were postfixed in 95 per cent ethanol at 60°C for 60 minutes (11).

C. FORMALIN: 10 per cent neutral formalin in phosphate buffer, pH 7.2, for 10 minutes at 4°C.

D. OSMIUM TETROXIDE: Zetterqvist's modification of the Palade fixative (12), 5 minutes at 4°C.

b) Staining Procedures

A. ACRIDINE ORANGE: Cells were rinsed in two changes of acetate buffer (0.1 M, pH 5.6), the first rinse for 5 minutes and the second 10 minutes, then stained for 15 minutes in 0.005 per cent acridine orange (Chroma-Gesellschaft, Stuttgart) in acetate.
buffer, and rinsed 3 or 4 times for 10 minutes each in acetate buffer (13). Stained cells were mounted in acetate buffer containing 20 per cent glycerol, sealed with Kronig's cement, and stored at 4°C.

b. fluorescent antibody: The indirect staining procedure was used (14). The γ-globulin fraction of rabbit anti-rat γ-globulin, fluorescein conjugated (Sylvania Chemical Company, Orange, New Jersey), was used in all studies. This was adsorbed twice with an acetone powder from beef liver (Sylvania Chemical Company, Orange, New Jersey). Rat anti-Mengo convalescent serum (heated at 56°C for 30 minutes) or a γ-globulin fraction prepared from this serum was used as a source of specific antibody. This was adsorbed with a suspension of frozen and thawed L cells to reduce staining of non-infected control cells.
c. Alkaline fast green stain was carried out according to the procedure of Alfert and Geschwind (15).
d. Bromphenol blue stain and the lysine blocking reactions were carried out according to the procedures described by Bloch and Hew (16, 17).
e. Sakaguchi reaction for arginine was carried out according to new modifications developed by Dr. A. Deitch (18).
f. The Millon reaction, as modified by Baker (19), was used to identify tyrosine.

c) Enzymes
The following enzymes were used: Ribonuclease—bovine pancreas (5 X crystallized)—Biochimica Boehringer; Deoxyribonuclease—bovine pancreas (1 X crystallized)—Mann Laboratories, New York City; Pepsin—(2 X crystallized)—Mann Laboratories.

3. Microscopy
All microscopy was done using the Zeiss Ultraphot II photomicroscope. Kodak Panatomic-X sheet film was used for black and white photomicrographs. Light microscopy and fluorescence microscopy employed an oil-immersion Apochromatic objective, 40 X/NA 1.0, with iris diaphragm. In general an objective NA of 1.0 was used for light microscopy and 0.6 for fluorescence microscopy in conjunction with a darkfield condenser. Appropriate excitation and barrier filters were used for fluorescence microscopy. Phase microscopy was done with a Neofluar objective, 40 X/NA 0.75, or a Neofluar oil immersion objective, 100 X/NA 1.30, using monochromatic light of λ = 546 mμ, obtained with an interference filter. Fixed cells were mounted in oil of refractive index 1.530 (R. P. Cargille Laboratories, New York City) and living cells in phosphate buffer containing 5 per cent horse serum.

RESULTS
1. Growth of Mengovirus in L-929 Cells
A growth curve obtained under the conditions of infection described under Materials and Methods is shown in Fig. 1. Time after start of infection was arbitrarily calculated from the end of the 30-minute adsorption period. New infectious virus appeared between 2 and 3 hours after infection and increased in amount up to 6 hours. By comparing total virus, i.e. that present in cells plus supernate, with virus in supernate only, it is clear that most virus is formed before an appreciable amount is released. Release started 3 hours after infection but even at 12 hours approximately 70 per cent of the infectious virus was still intracellular. Similar results were previously obtained with the mouse encephalomyelitis virus (7). In the experiment summarized in Fig. 1, the average number of cells per plate was 8.9 X 10⁵ (not a complete monolayer) and the average total amount of virus per plate was 8.0 X 10⁸ PFU. Therefore, the average yield of infectious virus per cell was 900 PFU in this experiment. The yield of infectious virus varied considerably in individual experiments, in some cases reaching 12,000 PFU per cell. The basis for this variation in yield is not known.

Hemagglutination tests on total virus yield are summarized in Table I. A comparison of hemagglutinin with infective virus indicated that an excess of hemagglutinating units was not found at late stages of infection, as is the case with influenza (20) or multiform viruses (21).

2. Alterations in Nucleic Acid During Viral Infection
At appropriate pH values, complexes of acridine orange with desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) fluoresce with different modes of decay, resulting in different fluorescence spectra. Although best recorded by color photography, significant changes in nucleic acids can be illustrated by black-and-white photography. Fig. 2 shows normal L cells fixed with acetate alcohol and stained with acridine orange. The nucleus fluoresced green, characteristic of DNA; the nucleoli red, characteristic of RNA; and the cytoplasm red, characteristic of RNA. The nucleolar-associated DNA was recognizable as areas surrounding the nucleoli and fluoresced deep green.

R. M. FRANKLIN Mengovirus in L-929 Cells 3
granular appearance of the nucleus was partly due to fixation artifacts (22).

The earliest change observed in infected cells was a subtle aggregation of nuclear DNA. In some cells this could be observed 1 hour after infection and was found in many cells 2 hours after infection. Profound cytopathic changes in the nuclei appeared 6 to 8 hours after infection (Figs. 3 and 4), and by 12 to 14 hours most cells were destroyed. There was a shrinkage in the nucleus accompanied by a change from round to kidney shape. At no time was any material having the staining characteristic of DNA observed to leave the nucleus of intact cells. At 10 to 12 hours some nuclei disintegrated resulting in release of DNA into the cytoplasm.

Two alterations in cytoplasmic RNA pattern were observed in Mengovirus-infected cells. The first cytoplasmic alteration was perinuclear accumulation of RNA as shown in Fig. 3. This was seen in

![Figure 1](https://example.com/figure1.png)

**Figure 1**

Growth of Mengovirus in L cells. Total virus (closed circles) refers to virus in cells plus that in the supernate, and released virus (open circles) refers to virus in the supernate.
Table I
Comparison of Total Hemagglutinating Units and Total Infectious Virus During the Growth of Mengovirus in L-929 Cells

<table>
<thead>
<tr>
<th>Time after infection</th>
<th>Hemagglutinating (HA) units/ml</th>
<th>Average PFU/ml</th>
<th>Ratio: PFU/HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1-2</td>
<td>$1.8 \times 10^4$</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>1-2</td>
<td>$9.2 \times 10^3$</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>1-2</td>
<td>$5.6 \times 10^3$</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>1-2</td>
<td>$5.4 \times 10^4$</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>$4.4 \times 10^7$</td>
<td>$1.4 \times 10^4$</td>
</tr>
<tr>
<td>5</td>
<td>64</td>
<td>$8.1 \times 10^7$</td>
<td>$1.3 \times 10^4$</td>
</tr>
<tr>
<td>6</td>
<td>236</td>
<td>$2.0 \times 10^8$</td>
<td>$7.9 \times 10^4$</td>
</tr>
<tr>
<td>7</td>
<td>236</td>
<td>$1.5 \times 10^8$</td>
<td>$6.1 \times 10^4$</td>
</tr>
<tr>
<td>8</td>
<td>256</td>
<td>$2.8 \times 10^8$</td>
<td>$1.1 \times 10^6$</td>
</tr>
<tr>
<td>10</td>
<td>256-512</td>
<td>$1.5 \times 10^8$</td>
<td>$3.0-6.0 \times 10^5$</td>
</tr>
<tr>
<td>12</td>
<td>256-512</td>
<td>$3.5 \times 10^8$</td>
<td>$0.7-1.4 \times 10^6$</td>
</tr>
</tbody>
</table>

After infection, some cells about 3 hours after infection. By 5 hours it was found in all cells as an intensely fluorescing area. One central cytoplasmic area did not seem to be involved. This area has not yet been identified. At 6 hours after infection a second structure was seen, a dense body which fluoresced bright red (Figs. 4, 5 a and b). This will be designated an inclusion. The number of cells containing inclusions increased to reach a maximum of about 14 per cent at approximately 11 hours. Inclusions were seen in cells at later times but the perinuclear RNA, as well as other cytoplasmic RNA, showed decreased staining intensity.

The nucleoli were studied carefully by determining their distribution in normal and infected cells. In order to see these RNA centers in the nucleus clearly, the fixed cells were digested with DNase (100 μg/ml, in distilled water containing $10^{-5}$ M Mg++) for 1 hour at 37°C. Ten groups of 100 uninfected cells were counted and single groups of 100 infected cells were counted at various times after infection. The percentage of cells having x number of nucleoli is tabulated in Table II. A standard deviation (σ) was determined for the 10 groups of uninfected cells. Infected cells were compared with controls by calculating the absolute value of the percentage difference (Δ) and also the number of standard deviations from the average value of uninfected cells (σ factor). A comparison of standard deviation factors and an average for all classes (number of nucleoli per nucleus) at each time after infection, up to and including 6 hours, indicates a variation of less than 1.5 standard deviations from uninfected cells. Only at 7 hours or later can there be a trend to fewer nucleoli per nucleus be observed. The actual trend may be more pronounced than reported here since only those nuclei which had not undergone a marked cytopathic change could be satisfactorily analyzed. In conclusion, there was no increase in nucleoli during the period of virus multiplication. The later decrease in nucleoli was probably associated with the cytopathic effect.

According to Davies (22), fixation by freeze-substitution leads to fewer artifacts than acetic alcohol fixation. Therefore, the above observations were repeated using freeze-substitution as a fixative. There was decreased granulation of nuclear DNA, but the changes in RNA were as described above.

3. Further Characterization of RNA in Mengovirus-Infected Cells

RNA of infected cells was further characterized by extraction procedures summarized in Table III. Only perinuclear RNA could be removed by ribonuclease digestion and, in some cases, a small fraction of this RNA was resistant to ribonuclease. In control experiments, RNA of uninfected cells was completely removed by ribonuclease. The perinuclear cytoplasm could be distinguished as a faintly autofluorescing area in the uninfected cell after RNase digestion. As is well known, the normal cell, spread out on a surface, has a high concentration of cytoplasmic constituents close to the nucleus. This probably accounts for the autofluorescence.

Virus-infected cells were treated with ribonuclease, deoxyribonuclease, distilled water, or isotonic phosphate buffer, pH 7.2. Only ribonuclease removed the perinuclear RNA and none of the treatments removed inclusion RNA. Fig. 6 shows a brightly fluorescing inclusion after RNase treatment. Extractions were also made with 1 M NaCl at 4°C since soluble RNA goes into solution under these conditions (23). The intensity of staining of either perinuclear or inclusion RNA did not change after such treatment. Unfortunately, it is difficult to decide whether soluble RNA could be extracted from fixed cells under such conditions, but since RNA of normal fixed cells is susceptible to RNase digestion, it should also be susceptible to alterations in ionic strength. Further experiments are in progress to test the efficiency of extraction of soluble RNA with 1 M NaCl. Tentatively, the
lack of solubility in 1 M NaCl of most of the RNA of the infected cell suggests that it is of molecular weight higher than that of soluble RNA.

The most plausible explanation for the resistance of inclusion RNA to RNase degradation is its possible enclosure in protein or close association with some protein. This hypothesis was strengthened by extracting both inclusion and perinuclear RNA with pepsin, which would digest the protective protein and thereby solubilize the RNA; and with potassium permanganate, which is known to degrade ribosomes (24) as well as RNA virus particles (25).

4. Characterization of Viral Antigen

Viral antigen was localized using fluorescent antibody. Infected cells were compared with uninfected cells, which had only a very weak cytoplasmic fluorescence. The high specificity of the staining system was due to the use of freeze-substitution fixation and to pre-adsorption of the specific antiserum with normal cellular antigens.

The earliest specific fluorescence was observed in some cells 2 hours after infection in the form of a well defined perinuclear area, similar in shape and extension to the perinuclear area in which RNA accumulated. As infection progressed, viral antigen localized in a perinuclear region was detected in increasing numbers of cells until all cells contained such antigen at 4 hours after infection. Perinuclear antigen is shown in Fig. 7. At 6, 8, and 10 hours after infection, increasing numbers of cells also contained deeply fluorescing areas of localized viral antigen in structures similar to the inclusions described above (Fig. 8). At no time was it possible to find inclusions in all cells. Inclusions were found maximally in about 20 per cent of the cells at 10 to 11 hours. Some cells also contained viral antigen in peripheral extensions of the cytoplasm. By 12 hours most cells were degenerating, but the remaining cells still contained...
TABLE II

Variation in Number of Nucleoli per Nucleus During Multiplication of Mengovirus in L cells

<table>
<thead>
<tr>
<th>No. of nucleoli</th>
<th>&lt;2</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>&gt;9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>0</td>
<td>0.1</td>
<td>1.34</td>
<td>7.51</td>
<td>25.57</td>
<td>30.08</td>
<td>20.56</td>
<td>11.87</td>
<td>2.80</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>0.80</td>
<td>1.34</td>
<td>4.56</td>
<td>5.19</td>
<td>4.88</td>
<td>4.68</td>
<td>1.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hr</td>
<td>0</td>
<td>0</td>
<td>2.7</td>
<td>4.5</td>
<td>39.3</td>
<td>28.6</td>
<td>14.3</td>
<td>8.9</td>
<td>0</td>
<td>1.8</td>
</tr>
<tr>
<td>Δ factor</td>
<td>0</td>
<td>0.1</td>
<td>1.4</td>
<td>3.0</td>
<td>13.7</td>
<td>3.3</td>
<td>6.3</td>
<td>4.2</td>
<td>2.80</td>
<td>1.8</td>
</tr>
<tr>
<td>s factor</td>
<td>&lt;1</td>
<td>1.5</td>
<td>2</td>
<td>3</td>
<td>&lt;1</td>
<td>1</td>
<td>&lt;1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hr</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8.0</td>
<td>27.0</td>
<td>29.0</td>
<td>28.0</td>
<td>6.0</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>Δ factor</td>
<td>0</td>
<td>0.1</td>
<td>1.3</td>
<td>0.5</td>
<td>1.4</td>
<td>1.1</td>
<td>7.4</td>
<td>1.3</td>
<td>2.80</td>
<td>2.0</td>
</tr>
<tr>
<td>s factor</td>
<td>&lt;1</td>
<td>1.5</td>
<td>2</td>
<td>3</td>
<td>&lt;1</td>
<td>1.5</td>
<td>&lt;1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 hr</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.9</td>
<td>18.7</td>
<td>40.5</td>
<td>20.8</td>
<td>6.9</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>Δ factor</td>
<td>0</td>
<td>0.1</td>
<td>1.3</td>
<td>2.4</td>
<td>6.9</td>
<td>10.4</td>
<td>0.2</td>
<td>1.9</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>s factor</td>
<td>&lt;1</td>
<td>1.5</td>
<td>2</td>
<td>1.5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 hr</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.8</td>
<td>30.3</td>
<td>29.4</td>
<td>15.7</td>
<td>10.8</td>
<td>3.9</td>
<td>0</td>
</tr>
<tr>
<td>Δ factor</td>
<td>0</td>
<td>0.1</td>
<td>1.3</td>
<td>2.3</td>
<td>4.7</td>
<td>0.7</td>
<td>4.9</td>
<td>6.1</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>s factor</td>
<td>&lt;1</td>
<td>1.5</td>
<td>2</td>
<td>1</td>
<td>&lt;1</td>
<td>1.5</td>
<td>&lt;1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 hr</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.4</td>
<td>10.4</td>
<td>25.5</td>
<td>32.0</td>
<td>11.3</td>
<td>6.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Δ factor</td>
<td>0</td>
<td>0.1</td>
<td>8.1</td>
<td>2.9</td>
<td>0.1</td>
<td>1.9</td>
<td>9.3</td>
<td>1.9</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>s factor</td>
<td>&lt;1</td>
<td>10.0</td>
<td>2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 hr*</td>
<td>5.0</td>
<td>0</td>
<td>8.9</td>
<td>27.7</td>
<td>20.8</td>
<td>18.8</td>
<td>11.9</td>
<td>4.0</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Δ factor</td>
<td>5.0</td>
<td>0.1</td>
<td>7.6</td>
<td>20.2</td>
<td>4.8</td>
<td>11.3</td>
<td>9.9</td>
<td>0.7</td>
<td>1.8</td>
<td>0.99</td>
</tr>
<tr>
<td>s factor</td>
<td>&lt;1</td>
<td>9.5</td>
<td>1</td>
<td>2</td>
<td>&lt;1</td>
<td>1</td>
<td>2</td>
<td>&lt;1</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>10 hr</td>
<td>0</td>
<td>2.9</td>
<td>9.7</td>
<td>25.2</td>
<td>24.2</td>
<td>23.3</td>
<td>9.7</td>
<td>4.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Δ factor</td>
<td>0</td>
<td>2.8</td>
<td>8.4</td>
<td>17.7</td>
<td>1.4</td>
<td>6.8</td>
<td>10.9</td>
<td>0.1</td>
<td>2.8</td>
<td>0</td>
</tr>
<tr>
<td>s factor</td>
<td>&lt;1</td>
<td>9</td>
<td>12</td>
<td>13</td>
<td>&lt;1</td>
<td>1.5</td>
<td>2</td>
<td>&lt;1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>12 hr‡</td>
<td>1.0</td>
<td>7.0</td>
<td>17.0</td>
<td>22.0</td>
<td>31.0</td>
<td>14.0</td>
<td>8.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Δ factor</td>
<td>1.0</td>
<td>6.9</td>
<td>15.7</td>
<td>14.5</td>
<td>5.4</td>
<td>16.1</td>
<td>12.6</td>
<td>4.7</td>
<td>2.8</td>
<td>0</td>
</tr>
<tr>
<td>s factor</td>
<td>21.5</td>
<td>19.5</td>
<td>11</td>
<td>1</td>
<td>3</td>
<td>2.5</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 5 per cent have no nucleoli.
‡ 1 per cent have one nucleolus.

viral antigen in the perinuclear area, in the inclusions, and at the cell periphery. No viral antigen was found in the nucleus at any stage of infection.

The viral antigen, especially in the inclusions, was further characterized using well known staining procedures. This was possible since viral antigen was present in high concentration in the inclusions. Indeed, these seemed to be areas containing crystalline arrays of virus (26). Bromphenol blue stain was used in conjunction with acetylation or oxidative deamination pretreatments. Most of the bromphenol blue staining was due to lysine (Figs. 9 and 10). Arginine was detected using a modification of the Sakaguchi reaction. The presence of tyrosine was demonstrated using Millon's reagent. Despite the presence of significant amounts of arginine and lysine, the viral protein did not seem to have an over-all basic character since it did not stain with alkaline fast green.

5. Description of Changes in Fixed and Living Cells Using Phase Contrast Microscopy

Cells fixed with osmium tetroxide appear very life-like by phase contrast microscopy (27). Both a
TABLE III

Effects of Various Reagents on the Staining of Nucleic Acid with Acridine Orange

L-929 cells were fixed either with acetic alcohol or by freeze-substitution, 11 hours after infection. + indicates removal of nucleic acid and − indicates resistance of nucleic acid.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Perinuclear RNA</th>
<th>Inclusion RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease (100 μg/ml in distilled water)</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Deoxyribonuclease (100 μg/ml in distilled water with 10⁻⁴ M Mg²⁺)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Distilled water</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>0.1 M phosphate buffer, isotonic, pH 7.2</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pepsin (0.002 per cent in 10 per cent acetic acid)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Potassium permanganate (0.4 per cent in distilled water)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 11 shows non-infected cells, spread on glass. Six hours after infection, an accumulation of perinuclear material in high concentration was evident (Fig. 12). This material may become rather granular by 8 hours (Fig. 13). Dense bodies first appeared at 6 hours and were present in many cells by 8 hours. They were clearly associated with a release of material from the cell as shown in the 7 minute sequence of Figs. 15 and 16. The cytopathic nuclear alterations discussed above are also clearly seen in these two figures. Many peripheral inclusion bodies were seen in cells at all later times (Figs. 14 and 17). Most of these structures protruded into the medium. It is easy to imagine that these structures may burst upon fixation, thus explaining the relatively small percentage of fixed cells having similar structures.

FIGURE 7
L cells infected with Mengovirus for 5 hours, stained with fluorescent antibody. Fixation: freeze-substitution. Antigen is present in a perinuclear area and there is no nuclear antigen. Any fluorescence of the nucleus is either non-specific or due to cytoplasmic antigen surrounding the nucleus. Magnification, 560.

FIGURE 8
L cells infected with Mengovirus for 10½ hours, stained with fluorescent antibody. Fixation: freeze-substitution. Antigen is present in the perinuclear area, in the cytoplasm in general, and in high concentration in peripheral inclusions. Magnification, 560.
FIGURE 9
L cells infected with Mengovirus for 12 hours, stained with bromphenol blue. Fixation: freeze-substitution followed by postfixation in 95 per cent alcohol. The cells do not appear sharp due to mismatching of refractive indices. Perinuclear accumulation of protein is clearly seen in several cells (arrows). Magnification, 720.

FIGURE 10
Same as Fig. 9 with acetylation prior to staining. Inclusions can be faintly seen in several cells (arrows).

FIGURE 11
Uninfected living L cells by phase contrast. Magnification, 560.

FIGURE 12
L cells infected with Mengovirus for 6 hours. An increase in density of the perinuclear region can be seen in some cells. Magnification, 560.

FIGURE 13
L cells infected with Mengovirus for 8 hours. The perinuclear region has an extremely granular appearance. Magnification, 560.

FIGURE 14
L cells infected with Mengovirus for 10 hours. Dense bodies, apparently corresponding to the inclusions, are seen in many cells. Magnification, 560.
Using monochromatic light and polaroid H-film, it was possible to utilize the phase contrast optics to construct a simple polarizing microscope. Living cells could be observed in phase contrast and then with "crossed Nicols." Neither the perinuclear area nor the inclusions of living cells showed any detectable birefringence.

**DISCUSSION**

The following sequence of cytochemical events has been observed in Mengovirus-infected L cells. Between 1 and 2 hours after infection there is subde granulation of nuclear DNA, but no increase in number of RNA centers (nucleoli). RNA accumulated in a perinuclear area between 3 and 5 hours after infection. Alterations in cytoplasmic RNA cannot be detected at earlier times, presumably due to the background of cytoplasmic RNA found in the normal cell. Most of the perinuclear RNA is susceptible to digestion by pancreatic ribonuclease. At 6 hours and later, RNA is also found in dense inclusions. This RNA is not digestible with RNase and is probably located in completed virus particles and is in the process of being released from the cell. The nucleus undergoes a series of degenerative changes and may disintegrate completely as the cell reaches its final pathological stages. After most of the virus is released, the cytoplasm is quite devoid of RNA.

Viral protein can be detected as early as 2 hours after infection in some cells. The ability to detect viral protein so early may be due to the extremely low background fluorescence with the fluorescent antibody technique. Viral antigen is first found in a perinuclear area and later in inclusions. Both of these areas seem to coincide with the areas of RNA accumulation. At no time is there any evidence for viral antigen in the nucleus. The probable localization of completed virus in dense areas just prior to release has been further supported by electron microscopy of Mengo-infected L cells (26).

The changes described above bear striking resemblance to the alterations in poliovirus-infected HeLa and monkey kidney cells (28-30). The earliest changes always occur in the nucleus and consist of condensation of chromatin (28, 30) and the appearance of acidophilic intranuclear inclusions (28) or may consist of merely a slight change in the nuclear membrane (29). Different changes occur in human amnion cells infected with poliovirus (31). In this case the earliest changes are loss of nucleolar staining intensity followed by disappearance of the nucleoli. In Mengo-infected L cells, nucleoli disappear only at a late stage of infection and there are no intranuclear inclusions at any stage of infection. Cytoplasmic eosinophilic bodies have been found in a perinuclear area in poliovirus-infected cells (28) and these could well correspond to the perinuclear RNA and viral protein described here. The appearance of peripheral nodules has been described for human amnion cells infected with poliovirus (31) and shrinkage of the cytoplasm with the formation of a peripheral hyaline zone in infected monkey kidney cells has also been described (32). The latter effect can be seen in late stages of Mengo infection (Fig. 17).

The appearance at about 5 hours after infection of cytoplasmic material having staining characteristics of RNA has been reported in polio-infected monkey kidney cells (33). No such material was found in Mengo-infected L cells until very late stages of cellular degeneration. Further data concerning this problem will be presented in a later paper.

The early morphological changes in the nucleus of polio-infected cells have led to the concept of nuclear participation in the multiplication of this RNA virus (28, 33, 34). The suggestion has been made that all RNA animal viruses may have an obligatory early nuclear phase and that the RNA may multiply in the nucleus (34). Nuclear changes, however, can also be the result of cytoplasmic dis-

**FIGURES 15 AND 16**

L cells infected with Mengovirus for 8 hours. Most cells in this field have peripheral inclusions. Fig. 16 was taken seven minutes after Fig. 15. These two figures illustrate the release of an inclusion (arrows in both figures). Magnification, 560.

**FIGURE 17**

L cells infected with Mengovirus for 11 hours. Peripheral inclusions are found in almost all cells at this time. Many inclusions which appear to be within the cell are actually at the surface of the cell. Magnification, 2200.
turbances (35). It is true that the early nuclear changes occurring in autolytic degeneration are different from those observed in polio- or Mengo-infected cells. Until more is known concerning the physiology and biochemistry of virus-infected cells during the latent period, it is difficult to evaluate the nuclear changes.

The author is grateful to Miss Joan Callender for her excellent technical assistance.

This study was aided by a grant from The National Foundation.

Received for publication, June 13, 1961.

BIBLIOGRAPHY

29. Dunnebacke, T. H., Correlation of the stage of cytopathic change with the release of poliomyelitis virus, *Virology*, 1956, 2, 399.


