Replication of Mengovirus

I. Effect on Synthesis of Macromolecules by Host Cell

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ABSTRACT

PLAGEMANN, PETER G. W. (Western Reserve University, Cleveland, Ohio), AND H. EARLE SWIM. Replication of mengovirus. I. Effect on synthesis of macromolecules by host cell. *J. Bacteriol.* **91**:2317-2326. 1966.—The replication of mengovirus was studied in two strains of Novikoff (rat) hepatoma cells propagated in vitro. The replicative cycle in both strains required 6.5 to 7 hr. Infection resulted in a marked depression of ribonucleic acid (RNA) and protein synthesis by strain N1S1-63. Inhibition of RNA synthesis was reflected by a decrease in the deoxyribonucleic acid (DNA)-dependent RNA polymerase activity of isolated nuclei. Mengovirus had no effect on either protein or RNA synthesis or on the DNA-dependent RNA polymerase activity of a second strain, N1S1-67. The time course of viral-induced synthesis of RNA by cells was studied in cells treated with actinomycin D. It was first detectable between 2.5 and 3 hr after infection and continued until 6.5 to 7 hr. The formation of mature virus was estimated biochemically by measuring the amount of RNA synthesized as a result of viral infection which was resistant to degradation by ribonuclease in the presence of deoxycholate. Approximately 70% of the deoxycholate-ribonuclease-resistant RNA was located in mature virus, and the remainder was double-stranded. The formation of mature virus began about 45 min after viral-directed (actinomycin-resistant) synthesis of RNA was detectable in the cell, and only about 18 to 20% of the total RNA synthesized was incorporated into virus. Release of virus from cells began about 1 hr after maturation was first detectable. Release of virus from cells was accompanied by a loss of a large proportion of their cytoplasmic RNA and protein.

Infection of mammalian cells with certain picornaviruses depresses the rate of synthesis of cellular ribonucleic acid (RNA) and protein (12, 16, 18, 19, 22, 28, 30, 36). In addition, infection of L cells with mengovirus (4) and of HeLa cells with poliovirus (17) results in a marked decrease in the deoxyribonucleic acid (DNA)-dependent RNA polymerase activity of the nucleus. The initial depression of synthesis of cellular RNA and protein is followed by an increase in the rate of synthesis of RNA and protein (12, 16, 24, 30, 36). The synthesis of viral RNA may not require a DNA template, because it is unaffected by actinomycin D in concentrations which inhibit the DNA-dependent synthesis of RNA in mammalian cells (27). This is also indicated by the observation that cell-free fractions from infected cells exhibit RNA polymerase activity which is

resistant to actinomycin D and not found in uninfected cells (5, 6). Actinomycin-resistant synthesis of viral RNA by protoplasts (20) and the appearance of viral-induced RNA polymerase activity (2, 15, 35) have been demonstrated in *Escherichia coli* infected with RNA phage. These observations also suggest that infection with RNA viruses results in the induction of a DNA-independent RNA polymerase in the host cell.

The present investigation was undertaken to obtain more information on the mechanism by which replication of viral RNA occurs in mammalian cells, with the use of a mengovirus-Novikoff hepatoma cell system as a model. This communication describes the general characteristics of this system and the results of studies of the effect of viral infection on the synthesis of RNA, DNA, and protein by Novikoff hepatoma cells. The data indicate that inhibition of host-cell synthesis of RNA and protein early in infection is not a prerequisite for the production of men-

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gavirus. The results also demonstrate that only a small proportion of the RNA synthesized as a result of infection is utilized for viral maturation.

MATERIALS AND METHODS

Materials. Materials were obtained as follows: sodium deoxycholate (DOC; enzyme grade) from Mann Research Laboratories, New York, N.Y.; methanol (reagent grade) from J. T. Baker Chemical CO., Phillipsburg, N.J.; *p*-dioxane (Eastman grade) and naphthalene (Eastman grade) from Eastman Organic Chemicals, Rochester, N.Y.; 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP; scintillation grade) from Packard Instrument Co., La Grange, Ill.; bovine serum albumin from Armour Pharmaceutical Co., Kankakee, Ill.; C^{14} -algal hydrolysate from Volk Radiochemical Co., Chicago, Ill.; uniformly labeled phenylalanine- C^{14} , uridine- H^3 , and uridine-2- C^{14} from New England Nuclear Corp., Boston, Mass.; unlabeled and labeled nucleoside triphosphates from Schwarz BioResearch, Orangeburg, N.Y.; phosphoenol pyruvate (PEP), pyruvate kinase, ribonuclease (type I from bovine pancreas), and deoxyribonuclease (type I from bovine pancreas) from Sigma Chemical Co., St. Louis, Mo.; pancreatic autolysate from Sheffield Chemical Co., Norwich, N.Y.; media 63 and 67 from Grand Island Biological Co., Grand Island, N.Y.; Hyflosupercel from Johns Manville, New York, N.Y. Actinomycin D and 5-fluorodeoxyuridine (FUDR) were gifts from A. E. Osterberg, Cancer Chemotherapy Section, National Institutes of Health, Bethesda, Md.

Buffer and cell culture media. Buffer (B1) contained the following (in millimoles): potassium chloride, 10; tris(hydroxymethyl) aminomethane (Tris)-HCl (pH 7.4), 10; and magnesium chloride, 1.5 (33). Earle's saline (ES) was prepared as described by Earle (10). SI47A had the same composition as SI03 (31) except that hydroxyproline was omitted and the concentration of glucose was increased to 15 mM. BM42 was composed of SI47A supplemented with 1 mg/ml of Pluronic F68 (32). BM42 was prepared from a dry powder (31). Medium 67 was composed of BM42 supplemented with 5% (v/v) calf serum and 2 mg/ml of pancreatic autolysate. Medium 63 contained 80% BM42 and 20% horse serum (v/v).

Cell culture. The N1S1 cells were established in 1959 (Swim, *unpublished data*) from the Novikoff strain (23) of rat hepatoma, and have been maintained in suspension culture according to procedures described by Swim and Parker (32). Strain N1S1-63 was obtained from a single cell and was propagated in medium 63. N1S1-63 does not grow in medium 67, but nutritional mutants which will proliferate under these conditions are present in the ratio of approximately 1 in 10^6 cells. Strain N1S1-67 was such a nutritional mutant isolated from N1S1-63 by selection in medium 67. Cells were enumerated by means of a Coulter counter (14). Vital staining was accomplished by mixing a sample of cell-suspension with 0.25 volume of 5% aqueous trypan blue. The proportion of nonviable (stained) cells in a culture was estimated by direct count in a hemacytometer. Stock cultures

were maintained in exponential growth by subculturing at 2-day intervals with an inoculum of 5×10^4 cells per milliliter.

Mengovirus. Mengovirus (8) does not multiply in N1S1 cells. A host-range mutant was isolated by passing this strain of virus for seven passages in a mixed culture of N1S1-63 and sarcoma 180 (Gerber and Swim, *unpublished data*). This strain of virus has been serially propagated in N1S1 for 40 passages during the course of this research. The concentration of virus was estimated by hemagglutination (HA) assay (11) or by infectivity titrations in N1S1 cells maintained in roller tubes. Ten-fold dilutions of virus were prepared in ES, and 0.1 ml was added to each of four tubes containing 10^5 cells in 2 ml of fresh BM42. The cells were incubated at 37 C and after 4 days were examined for cytopathic changes. The $TCID_{50}$ was calculated by the method of Reed and Muench (26).

Virus was propagated by exposing cells in BM42 at a density of 10^7 per milliliter to 5–50 $TCID_{50}$ per cell of virus for 30 min at 37 C. The suspension was then diluted to 10^6 to 2×10^6 cells per milliliter and incubated at 37 C on a rotary shaker for 12 to 24 hr. Cellular debris was removed by centrifugation at $4,000 \times g$ for 30 min or by passage of approximately 250 ml/min through a Sharples centrifuge (30,000 rev/min). The supernatant fluid contained between 10^8 and 10^9 $TCID_{50}$ per ml and was stored at –20 C. Cells for experiments were infected in BM42 with 10 to 50 $TCID_{50}$ per cell as described in the foregoing.

Cell fractionation. All steps were conducted at 0 to 4 C. Cells were removed from the medium by centrifugation, at $500 \times g$ for 5 min, and were washed in 10 volumes of ES. The cells were resuspended in 8 to 10 volumes of B1 and allowed to stand at 0 C for 15 min. The cells were disrupted by 8 to 10 strokes with a Dounce homogenizer. Microscopic examination showed that over 95% of the cells were broken by this procedure. The nuclei were sedimented at $800 \times g$ for 10 min, and the supernatant fluid was referred to as the postnuclear fraction.

Chemical determinations. Protein was estimated by the method of Lowry et al. (21). RNA and DNA were determined in samples of cell fractions after precipitation with equal volumes of 1 N perchloric acid (PCA) at 0 C. The precipitate was washed three times with cold 0.5 N PCA and heated in 0.5 N PCA at 70 C for 30 min. The hydrolysate was centrifuged at $2,000 \times g$ for 10 min, and the resulting supernatant fluid was analyzed for RNA by the orcinol method (1) and for DNA by the diphenylamine method (9). All values for RNA were corrected for the presence of DNA by the method of Schneider (29). Bovine serum albumin, yeast RNA, and thymus DNA served as standards.

Assay for DNA-dependent RNA polymerase. Samples of 0.2 ml of nuclear fraction containing 0.6 to 1.5 mg of protein were incubated with 0.1 ml of saturated $(NH_4)_2SO_4$ (pH 8.2) and 0.4 ml of a solution containing (in micromoles): magnesium acetate, 5; Tris-HCl buffer (pH 8.2), 50; adenosine triphosphate (ATP), cytidine triphosphate (CTP), and uridine triphosphate (UTP), each 0.06; PEP, 4; 8 μ g of pyruvic kinase; and 0.1 μ C of guanosine triphosphate (GTP)- C^{14} (13 μ C/ μ mole) or 1 μ C of GTP- H^3 (1,700 μ C/ μ mole). The

mixture was incubated at 37 C for 30 min and then analyzed for radioactivity incorporated into acid-insoluble material.

Determination of acid-insoluble radioactivity. Samples of 0.5 to 1 ml were chilled to 0 C and mixed with 1 ml of 0.1 M sodium pyrophosphate containing 2 mg of bovine serum albumin. The acid-insoluble material was precipitated at 0 C by the addition of 2.5 ml of 1 N PCA containing 25 mg of Hyflosupercel. The mixture was allowed to stand for 10 min, and the precipitate was removed by centrifugation at $1,000 \times g$ for 5 min. The precipitates were washed three times in 5 ml of 0.5 N PCA at 0 C and then suspended in 1 ml of 0.5 N trichloroacetic acid and heated for 30 min at 70 C. After cooling, the samples were transferred quantitatively to scintillator bottles and assayed for radioactivity.

Uridine incorporation. Details of the conditions are described in the appropriate experiments. Samples were analyzed for acid-insoluble radioactivity as described earlier.

Determination of deoxycholate-ribonuclease-resistant RNA. Samples of labeled cells were treated with 1% DOC and 20 μ g/ml of ribonuclease at 37 C for 20 min, and the acid-insoluble material remaining was referred to as DOC-(ribonuclease)-resistant RNA.

Incorporation of labeled amino acids. Duplicate 3-ml samples of cell suspension were pulse-labeled with C^{14} -amino acids as described in the appropriate experiments. A 1-ml amount of a 0.2% solution of bovine serum albumin and 3 ml of 1 N PCA containing 30 mg of Hyflosupercel were then added, and the mixture was heated at 70 C for 30 min. The mixture was cooled, and the acid-insoluble material was recovered by centrifugation at $2,500 \times g$ for 10 min. The pellet was washed twice with 5 ml of 0.5 N PCA, once with 5 ml of trichloroacetic acid, and finally dissolved in 1 ml of formic acid, and analyzed for radioactivity.

Radioactivity determinations. Aqueous samples containing 0.5 to 1.0 ml were added to 15 ml of scintillation fluid prepared by adding 200 ml of methanol, 125 g of naphthalene, 7.5 g of PPO, and 0.75 g of POPOP to 1 liter of *p*-dioxane. The radioactivity was determined with a Packard automatic three-channel Tricarb liquid scintillation spectrometer. All values were corrected for background.

Sucrose density gradients. Linear sucrose density gradients were prepared in B1 as described by Britten and Roberts (7).

RESULTS

Growth curve of mengovirus in N1S1-67 cells. A typical growth curve of mengovirus in N1S1-67 cells is illustrated in Fig. 1. Mature virus appeared in the cells at approximately 4 hr after infection and began to be released into the medium about 0.5 to 1 hr later. Viral multiplication was complete at 6 to 7 hr. An increase in trypan blue-positive cells was evident by 6 hr, and by 8 to 9 hr 95% of the cells were positive. Similar growth curves were observed in experiments performed with strain N1S1-63.

FIG. 1. Formation of cell-associated and extracellular mengovirus as a function of time. N1S1-67 cells were infected with 25 TCID₅₀ of mengovirus per cell, and the cells from 2-ml samples were collected by centrifugation at indicated intervals thereafter. The supernatant fluid was assayed for extracellular (EC) virus. The pellet was suspended in 2 ml of BM42, was frozen and thawed four times, and was then assayed for cell-associated (CA) virus.

Effect of viral infection on nucleic acid and protein composition of cells. The data presented in Fig. 1 show that mature virus begins to appear extracellularly within 1 hr after its formation and about 2 hr before viral maturation ceases. It was of interest to determine whether the release of virus from the cells is accompanied by a release of cellular components. Such losses could contribute to the rapid death of the host cell which accompanies viral replication. The results presented in Fig. 2 show that infected cells began to lose their cytoplasmic RNA and protein between 4 and 5 hr after infection, and this loss was substantial before viral replication ceased between 6 and 7 hr. Viral replication, on the other hand, had little effect on the DNA, RNA, and protein content of the nuclear fraction. Microscopic examinations showed that the observed increase in the amount of RNA and protein in the nuclear fraction during the terminal stages of viral replication probably resulted from increased contamination of the nuclei with cytoplasmic constituents during this period. The initial rise in protein and RNA content of the postnuclear fraction probably resulted from residual cell growth, since uninfected control cultures showed a similar increase. Poliovirus also causes a reduction in the amount of RNA and protein of HeLa cells without affecting their DNA content (28).

FIG. 2. Protein, RNA, and DNA content of nuclear and postnuclear fractions as a function of time after infection. In this instance, cells were disrupted in 20 volumes of B1. The nuclei were washed once in B1 and the wash fluid was pooled with the postnuclear fraction. The values indicated are for 10^7 cells based on count at zero-time.

Effect of viral infection on RNA synthesis by host cell. The effect of infection with mengovirus on the rate of uridine incorporation by N1S1-63 and N1S1-67 cells is illustrated in Fig. 3. The results presented in Fig. 3A show that the rate of uridine incorporation into acid-insoluble material by N1S1-63 cells began to decline immediately after infection, and reached a minimal value at 2.5 hr. Subsequently, the rate began to increase and reached a maximal level at 5 hr. This increase reflects the production of viral RNA as shown by the curve for uridine incorporation in the presence of actinomycin D. The initiation of actinomycin-resistant synthesis of RNA coincides with the production of viral-induced RNA polymerase (25). The data presented in Fig. 3B demonstrate that infection of N1S1-67 with mengovirus did not cause an initial depression in the rate of uridine incorporation as was observed

with N1S1-63. The rate of RNA synthesis was identical in both uninfected and infected N1S1-67 cells during the first 2.5 hr. A comparison of the results obtained with infected and uninfected cells reveals that viral-induced synthesis of RNA was apparent only as a small increase in the overall rate of uridine incorporation. It became clearly detectable only when RNA synthesis by the host cell was eliminated by treatment with actinomycin D. The data in Fig. 3 also show that the viral-

FIG. 3. Effect of mengovirus on RNA synthesis by N1S1-63 and N1S1-67 cells. Samples of 1 ml containing 10^6 N1S1-63 cells were pulse-labeled with $0.04 \mu\text{mole}$ of uridine- 2-C^{14} ($0.5 \mu\text{C}/\mu\text{mole}$) for 25 min. Comparable pulses with N1S1-67 were performed with $0.04 \mu\text{mole}$ of uridine- H^3 ($10 \mu\text{C}/\mu\text{mole}$). Samples were analyzed for acid-insoluble radioactivity, and each point on the curves represents the average value for duplicate determinations and is placed at the midpoint of the period of labeling. In each experiment, 0.5 mM FUDR was added at 0.5 hr. Actinomycin D was added at 0.5 hr in a concentration of $1 \mu\text{g}/\text{ml}$ where indicated.

induced synthesis of RNA followed a similar time course in both strains of cells. Variations were observed in the time course of viral-induced synthesis of RNA in infected cells. In some instances, it began as early as 2.5 hr after infection and stopped at 6 hr, whereas in other experiments the actinomycin-resistant synthesis of RNA began at 3 hr and continued until 7 hr.

Effect of viral infection on DNA-dependent RNA polymerase as function of host cell. The decrease in the rate of uridine incorporation by N1S1-63 cells after infection with mengovirus was accompanied by a marked reduction in the DNA-dependent RNA polymerase activity of isolated nuclei (Fig. 4). The overall results of several experiments similar to that presented in Fig. 4 indicated that the RNA polymerase activity of N1S1-63 cells began to decline consistently within the first 1.5 hr after infection, and only 10 to 20% of the original activity remained after 4 to 6 hr. Similar experiments with N1S1-67 cells, on the other hand, showed that the activity of

the host RNA polymerase was not affected significantly throughout the period of viral replication (Fig. 4). Ammonium sulfate was used in the assays for the DNA-dependent RNA polymerase (Fig. 4), because it eliminated interference by the viral-induced RNA polymerase by inhibiting its activity (25). Ammonium sulfate also stimulates the activity of the DNA-dependent RNA polymerase activity of isolated nuclei (4, 13).

Effect of infection on protein synthesis by different strains of Novikoff cells. The rate of incorporation of C^{14} -amino acids into protein was identical in both uninfected and infected N1S1-67 cells for the first 3 hr after infection (Fig. 5B). Viral-induced synthesis of protein became apparent between 4 and 6 hr after infection by a small increase in the rate of amino acid incorporation in infected cells over that observed in uninfected controls during the same period. The increased rate of protein synthesis in infected cells occurred at the same time as the viral-induced synthesis of RNA (Fig. 3). The loss in capacity of infected cells to synthesize RNA and protein coincided with an increase in the number of nonviable cells in the culture (Fig. 5B). The increased rate of protein synthesis which was observed after uninfected cells were washed and resuspended in BM42 was due to cellular multiplication which occurred under these conditions. The results obtained with N1S1-63, on the other hand, indicate that the rate of protein synthesis in infected cells was considerably less than that of uninfected cells (Fig. 5A). It will be noted that viral infection prevented the increase in rate of amino acid incorporation during the first 3 hr without altering the initial rate substantially. An increase in the initial rate of protein synthesis was observed only during the period (4 to 7 hr) of viral production.

Time course of actinomycin-resistant synthesis of RNA and its incorporation into mature virus. The following experiments were undertaken to determine the temporal relationship between the viral-induced synthesis of RNA in infected cells and the incorporation of the RNA into mature virus. The incorporation of RNA into virus was determined by utilizing the fact that the RNA in virus is resistant to degradation by ribonuclease in the presence of DOC. Infected cells were treated with actinomycin D and FUDR and then exposed to uridine- H^3 (Fig. 6). Under these conditions, uridine is almost exclusively utilized for viral-induced synthesis of RNA. Samples of cell suspension were analyzed for total acid-insoluble radioactivity and DOC-ribonuclease-resistant radioactivity at various intervals after the addition of labeled uridine. The results showed that

FIG. 4. Effect of viral infection on DNA-dependent RNA polymerase as function of host cell. Cells were infected with 50 TCID₅₀ per cell and their nuclei were assayed for DNA-dependent RNA polymerase at indicated intervals thereafter. Reaction mixture contained, in 0.7 ml: 5 μ moles of magnesium acetate, 5 μ moles of Tris-HCl buffer (pH 8.2), 4 μ moles of PEP, 8 μ g of pyruvate kinase, 0.06 μ mole each of ATP, CTP, and UTP, 1 μ c of GTP- H^3 (1,700 μ c/ μ mole), 0.1 ml of saturated $(NH_4)_2SO_4$ (pH 8.2), and nuclear fraction containing 1 mg of protein. Average values for duplicate determinations were plotted.

FIG. 5. Effect of viral infection on the rate of protein synthesis as a function of host cell. Cells were suspended at 10^6 per milliliter in a mixture composed of equal volumes of ES and BM42. The proportion of cells stainable with trypan blue was determined at the indicated times. Duplicate 3-ml samples were pulse-labeled as follows: NISI-63 cells were exposed to $0.01 \mu\text{C}$ of uniformly labeled phenylalanine- C^{14} ($10 \mu\text{C}/\mu\text{mole}$) for 60 min; NISI-67 cells were treated with $0.1 \mu\text{C}$ of algal hydrolysate- C^{14} ($163 \mu\text{C}/\text{mg}$) for 10 min. In each instance, the cells were recovered and assayed for acid-insoluble radioactivity. The points are placed at the midpoint of the labeling period.

the actinomycin-resistant synthesis of RNA began at about 2.5 hr after infection, proceeded at a constant rate between 3.5 and 5.5 hr, and declined thereafter. These data are in accord with the results of experiments employing pulse labeling (Fig. 3). The incorporation of radioactivity into DOC-ribonuclease-resistant material relative to the total varied with time. During the initial phase between 2.5 and 3.5 hr after infection, the DOC-ribonuclease-resistant RNA represented about 8% of the total RNA synthesized, whereas in the second phase between 4 and 6 hr it represented approximately 25%. The DOC-ribonuclease-resistant RNA formed between 2.5 and 3.5 hr is largely double-stranded RNA which is also resistant to ribonuclease under the conditions employed (Plagemann and Swim, *in preparation*). Between 60 and 75% of the DOC-ribonuclease-resistant RNA formed after 3.5 hr was found to be in mature virus, and the remainder in double-stranded RNA (Fig. 7 and Table 1). The temporal relationships of viral formation measured in this way agree with the results obtained by infectivity titrations and HA determinations (Fig. 1).

Proportion of DOC-ribonuclease-resistant RNA in mature virus. The data presented in Fig. 7 illustrate a sucrose density gradient profile of DOC-ribonuclease-resistant RNA synthesized between 4.5 and 5.5 hr after infection. The radio-

activity profile shows that the DOC-ribonuclease-resistant RNA was largely associated with two peaks. A comparison of the profiles for optical density and for radioactivity illustrates that one peak (approximately 150S) sedimented faster than the 73S ribosomes and the other at a slower rate. The HA activity was found largely in the 150S peak. The small amount of HA activity at the top of the gradient probably represented virus complexed with lipid material solubilized by the DOC (Hoshino and Swim, *unpublished data*). Experiments similar to that illustrated in Fig. 7 were performed to ascertain the relative proportion of DOC-ribonuclease-resistant RNA which is present in virus. To accomplish this, samples from different regions of the gradient were pooled as illustrated in Fig. 7, and the RNA in each of these major fractions was isolated by extraction with hot phenol-sodium dodecylsulfate and analyzed for single and double-stranded RNA as described by Plagemann and Swim (*in preparation*). The results demonstrated that fraction I (150S peak) contained exclusively single-stranded RNA with sedimentation characteristics (37S) identical to that extracted from purified virus. The RNA extracted from fraction II was found to be double-stranded (20S). Fraction III from the top of the gradient contained both double-stranded and viral RNA in a ratio of about 9:1. The results of several such experiments performed

at various times are summarized in Table 1. The DOC-ribonuclease-resistant RNA synthesized prior to 3.5 hr was almost exclusively double-stranded. After this time, however, approximately 70% of the DOC-ribonuclease-resistant RNA was located in mature virus, and the remainder was double-stranded. It will be noted that the values obtained with the postnuclear fractions were the same as those for whole cells. The combined data in Fig. 7 and Table 1 also demonstrated that double-stranded RNA accumulated from the time the synthesis of viral-induced RNA was initiated until the end of the replicative cycle.

DISCUSSION

The results of these experiments have shown that infection of strain N1S1-67 Novikoff cells with mengovirus does not result in a significant depression of the normal rate of synthesis of RNA and protein. The same strain of virus, however, inhibits the synthesis of both RNA and

FIG. 6. Actinomycin D-resistant synthesis of RNA as function of time after infection. At the times indicated, $1 \mu\text{g/ml}$ of actinomycin D, $0.5 \mu\text{mole/ml}$ of FUdR, and $6 \times 10^{-3} \mu\text{mole/ml}$ of uridine- H^3 ($350 \mu\text{C}/\mu\text{mole}$) were added to a suspension of infected cells at 1.5×10^6 per milliliter. Cells from 1-ml samples were assayed in duplicate for total acid-insoluble radioactivity and for acid-insoluble label resistant to treatment with DOC-ribonuclease.

FIG. 7. Sucrose density gradient analysis of DOC-ribonuclease-RNA. Infected cells at 1.5×10^6 per milliliter were treated with $2 \mu\text{g/ml}$ of actinomycin D and $0.5 \mu\text{mole/ml}$ of FUdR at 3.75 hr after infection, and with $2.7 \times 10^{-3} \mu\text{mole/ml}$ of uridine- 2-C^{14} ($30 \mu\text{C}/\mu\text{mole}$) from 4.5 to 5.5 hr. A postnuclear fraction was prepared, and DOC and ribonuclease were added to a final concentration of 1% and $1 \mu\text{g/ml}$, respectively, and the mixture was incubated for 15 min at 25°C . A 2-ml amount of treated postnuclear fraction was layered over a 0.5 to 1.2 M linear sucrose gradient prepared in B1 and centrifuged at 25,000 rev/min in a SW 25 rotor for 75 min. Twenty-drop fractions were collected and assayed for HA, C^{14} , and optical density at 260 mμ. Fractions were pooled as indicated by the Roman numerals in the graph for analysis of the proportion of single- and double-stranded RNA molecules.

protein by N1S1-63 (Fig. 3 and 5). Inhibition of RNA synthesis is accompanied by a decrease in the DNA-dependent RNA polymerase of the nucleus (Fig. 4). Inhibition of RNA and protein synthesis has been observed in Krebs ascites cells infected with EMC virus (22), in L cells infected with mengovirus (12, 19) or with Me virus (16, 30), and in HeLa cells (18, 28, 36) infected with poliovirus. The question of whether there is any direct relationship between the inhibition of RNA and protein synthesis has not been satisfactorily answered. A comparison of the data presented in Fig. 3 and 5 reveals that viral infection has a substantially greater effect on the synthesis of RNA than on protein. It will be noted that the rate of synthesis of protein by uninfected cells continues to increase throughout the period of

TABLE 1. Distribution of DOC-ribonuclease-resistant RNA between virus and double-stranded RNA as function of time*

Period of labeling (hr after infection)	Distribution of DOC- ribonuclease-resistant RNA as per cent of total	
	Virus	Double-stranded RNA
1.75-3†	9	91
1.75-3.75	11	89
4-6	73	27
4-5.5	66	34
4.5-5.5	71	29
4.5-5.25	80	20
4.75-5.5	72	28
4.75-5.5†	75	25
5-5.75	65	35

* Infected cells at 1.5×10^6 per milliliter were treated with 2 $\mu\text{g}/\text{ml}$ of actinomycin D and 0.5 $\mu\text{mole}/\text{ml}$ of FUDR 30 to 45 min prior to the addition of labeled uridine. Cells were labeled with uridine- H^3 (100 $\mu\text{C}/\mu\text{mole}$) or uridine- $2-C^{14}$ (25 $\mu\text{C}/\mu\text{mole}$) in concentrations ranging from 8×10^{-3} to 20×10^{-3} mM for the period indicated. Postnuclear fractions were prepared and treated with 1% DOC and 1 to 5 $\mu\text{g}/\text{ml}$ of ribonuclease for 15 to 20 min at 25 C. Individual postnuclear fractions were then analyzed for viral and double-stranded RNA either directly or after density gradient centrifugation (Fig. 7) as described by Plagemann and Swim (*in preparation*). Each set of values represents a separate experiment.

† Whole cells were lysed with 1% DOC and treated with 20 $\mu\text{g}/\text{ml}$ of deoxyribonuclease and 5 $\mu\text{g}/\text{ml}$ of ribonuclease for 20 min at 37 C. The mixture was then analyzed for double-stranded and viral RNA.

observation, whereas the initial rate declines 25% during the first 3 hr in infected cells. There is a similar decline in the rate of protein synthesis when N1S1-63 cells are exposed to actinomycin D (Beard and Swim, *unpublished data*). This information, coupled with the data in Fig. 3, 4, and 5, is consistent with the tentative conclusion that the effects of mengovirus on protein synthesis by N1S1-63 cells are secondary, and that the primary effect is inhibition of the DNA-dependent synthesis of RNA. Viral-induced suppression of messenger RNA (mRNA) synthesis would not account, however, for the results reported for other systems. For example, Zimmerman et al. (36) found that the rate of protein synthesis in poliovirus-infected HeLa cells began to decline shortly after infection, but RNA synthesis was unaffected for 2 hr. Franklin and Baltimore (12) observed that the rate of protein synthesis in L cells was affected more rapidly and to a greater

extent by infection with mengovirus than by treatment with actinomycin D. Similarly, viral infection has been reported to accentuate the effect of actinomycin D on protein synthesis by HeLa cells infected with poliovirus (18) and by L cells infected with Me virus (16). The data available, therefore, suggest that more than one mechanism may be operative in inhibition of protein synthesis by RNA viruses in mammalian cells. There is more general agreement, however, that poliovirus and mengovirus cause a reduction in the activity of the DNA-dependent RNA polymerase of the host cell (Fig. 4; 4, 17). The mechanism of viral-induced inhibition of RNA synthesis is not understood, but Baladin and Franklin (3) recently presented evidence that a protein may be synthesized in L cells after infection with mengovirus, which inhibits the DNA-dependent RNA polymerase of isolated nuclei. It has been suggested that the information for the synthesis of this and possibly other inhibitory substances resides in the viral RNA. The finding that mengovirus causes the inhibition of RNA synthesis by one strain of Novikoff cells but not by another (Fig. 3) suggests alternatively that the inhibition of the DNA-dependent RNA polymerase represents a variable host response. The results obtained with two strains of N1S1 cells also indicate that the inhibition of RNA synthesis by the host cell is not an essential feature in the replication of mengovirus. It is of interest in this connection that the DNA-dependent RNA polymerase activity of *E. coli* is not influenced by infection with an RNA phage (35). It is anticipated that this difference between strains N1S1-63 and N1S1-67 in response to infection with mengovirus will provide a useful model for future studies of regulatory processes in mammalian cells.

The formation of mature virus can be estimated biochemically by measuring the amount of labeled uridine incorporated into RNA which resists degradation by ribonuclease in the presence of DOC. This accounts for approximately 25% of the viral-directed RNA which is synthesized (Fig. 6). It has been shown that 60 to 75% of the DOC-ribonuclease-resistant RNA formed between 4 and 6 hr after infection is located in mature virus, and that the remainder is double-stranded RNA (Fig. 7 and Table 1). The DOC-ribonuclease-resistant RNA formed before viral maturation begins is double-stranded RNA (Table 1). The formation of mature virus begins about 45 min after viral directed (actinomycin-resistant) synthesis of RNA is detectable by the procedures employed (Fig. 1, 3, 6, and Table 1). The intervening period is probably required for

the initiation of synthesis of viral protein. Only 18 to 20% of the total RNA synthesized by Novikoff cells as the result of viral infection is incorporated into mature virus. It has been reported that other cell-virus systems utilize a larger proportion of RNA synthesized for viral maturation than observed with the mengovirus-Novikoff system. Zimmerman et al. (36) reported that 65% of the labeled uridine incorporated into acid-insoluble material by HeLa cells infected with poliovirus in the presence of actinomycin D was resistant to ribonuclease. These investigators equated ribonuclease resistance with mature virus, and the contribution of double-stranded RNA to this value is unknown. It is of interest in this connection that ribonuclease treatment of cell-free extracts from infected Novikoff cells in the presence of DOC solubilizes about twice as much RNA as in its absence. In experiments with Me virus-infected L cells, Hausen and Verwoerd (16) recovered 45% of the radioactivity incorporated between 3.5 and 6.5 hr after infection in virus. Although different systems vary with respect to the proportion of RNA incorporated into virus, the overall data indicate that the utilization of RNA for maturation of picornaviruses is either a very inefficient process or that much of it is made for another purpose. Evidence will be presented later (Hoshino, Plagemann, and Swim, *in preparation*) that a large proportion of the RNA synthesized by Novikoff hepatoma as a result of infection with mengovirus probably does not represent a precursor pool for viral maturation. This RNA is associated with polysomes, and may function as messenger for the synthesis of capsid protein.

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