JOURNAL OF VIROLOGY, May 1968, p. 461-473
Vol. 2, No. 5

Printed in U.S.A.

Mengovirus Replication in Novikoff Rat Hepatoma and Mouse L Cells: Effects on Synthesis of Host-Cell Macromolecules and Virus-specific Synthesis of Ribonucleic Acid

PETER G. W. PLAGEMANN

Department of Microbiology, Medical School, University of Minnesota, Minneapolis, Minnesota 55455

Received for publication 17 January 1968

Novikoff cells (strain N1S1-67) and L-67 cells, a nutritional mutant of the common strain of mouse L cells which grows in the same medium as N1S1-67 cells, were infected with mengovirus under identical experimental conditions. The synthesis of host-cell ribonucleic acid (RNA) by either type of cell was not affected quantitatively or qualitatively until about 2 hr after infection, when viral RNA synthesis rapidly displaced the synthesis of cellular RNA. The rate of synthesis of protein by both types of cells continued at the same rate as in uninfected cells until about 3 hr after infection, and a disintegration of polyribosomes occurred only towards the end of the replicative cycle, between 5 and 6 hr. The time courses and extent of synthesis of single-stranded and double-stranded viral RNA and of the production of virus were very similar in both types of cells, in spite of the fact that the normal rate of RNA synthesis and the growth rate of uninfected N1S1-67 cells are about three times greater than those of L-67 cells. In both cells, the commencement of viral RNA synthesis coincided with the induction of viral RNA polymerase, as measured in cell-free extracts. Viral RNA polymerase activity disappeared from infected L-67 cells during the period of production of mature virus, but there was a secondary increase in activity in both types of cells coincidental with virus-induced disintegration of the host cells. Infected L-67 cells, however, disintegrated and released progeny virus much more slowly than N1S1-67 cells. The two strains of cells also differed in that replication of the same strain of mengovirus was markedly inhibited by treating N1S1-67 cells with actinomycin D prior to infection; the same treatment did not affect replication in L-67 cells.

Results from previous studies (20–24) on the replication of mengovirus in Novikoff rat hepatoma cells (strain N1S1-67) differ in a number of important points from those obtained in similar studies with L cells (4, 10). Infection of L cells, for instance, results in a rapid inhibition of the synthesis of host-cell ribonucleic acid (RNA) and protein (4, 10), whereas in N1S1-67 cells both processes appear not to be affected by mengovirus infection, at least until the beginning of synthesis of viral RNA and protein (21). Although infection of both types of cells leads to the formation of a cytoplasmic RNA-dependent RNA polymerase after infection (3, 22), the time course of its formation, as measured in crude cell-free extracts, seems to differ markedly in L and Novikoff cells. In both types of cells, there is an initial rapid increase in virus-induced RNA polymerase activity between 2 and 4.5 hr after infection (3, 20, 22). In Novikoff cells, this is followed by a secondary two- to three-fold increase in activity between 5 and 7 hr (20, 22), whereas results from preliminary experiments (*unpublished*) indicated that, subsequent to the initial increase, the RNA polymerase activity rapidly disappears from infected L cells. In addition, we found that treatment of N1S1-67 cells with actinomycin D several hours prior to infection completely inhibited the induction of viral RNA polymerase in Novikoff cells and, consequently, the production of virus (23), whereas Franklin and Baltimore (10) reported that actinomycin D has no effect on the replication of mengovirus in L cells, even when administered 24 hr prior to infection. It was not clear from the various experiments whether the

differences in results with the two mengovirus-cell systems were a characteristic of the cell type or the virus strain or were due to differences in experimental procedure. The present investigation was undertaken to answer this question and to compare further the replication of mengovirus in these two types of cells. A nutritional mutant of L cells (L-67) which grows in the same medium as N1S1-67 cells was employed in these experiments. The cells were infected under identical conditions with the same strain of mengovirus.

MATERIALS AND METHODS

Materials. Materials were obtained as follows: ^{3}H-uridine and ^{3}H-amino acid mixture from New England Nuclear Corp., Boston, Mass.; ^{3}H-guanosine triphosphate (GTP) and unlabeled nucleoside triphosphates from Schwarz Bio Research, Orangeburg, N.Y. Actinomycin D was a gift from Merck Sharp and Dohme, Rahway, N.J., and 5-fluorodeoxyuridine (FUdR), from Hoffman-La Roche, Inc., Nutley, N.J. Sources of other materials have been stated previously (21).

Medium 67 was composed of basal medium 42 (BM42; 21) supplemented with 5% (v/v) calf serum and 2 mg of pancreatic autolysate per ml (21). Medium 63 contained 80% BM42 and 20% horse serum (21). Eagle's medium was composed of Eagle's basal medium with Earle's salts (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 17% (v/v) calf serum.

Cell culture and virus infection. Novikoff rat hepatoma cells (strain N1S1-67) and mouse L cells (strain L-67) were grown in suspension culture in medium 67 as described previously (21). The strain L-67 was isolated by selection in medium 67 from a strain of L cells obtained from K. G. Brand (5). The latter was maintained in monolayer culture in Eagle's medium. The generation times of N1S1-67 and L-67 cells in medium 67 at 37 C were 13 and 38 hr, respectively. N1S1-67 cells grew to a maximal concentration of about 3×10^6 cells per ml and L-67 cells, to 1.2×10^6 cells per ml.

The strain of mengovirus employed is a host-range mutant which replicates in Novikoff cells (21). Stock suspensions were prepared by propagating the virus in N1S1-67 cells as described previously (21). Methods for the enumeration of cells, vital staining with trypan blue, and the assay of virus concentration by infectivity and hemagglutination (HA) titrations have been described previously (21).

For experiments, cells were harvested during the exponential phase of growth: N1S1-67 cells between 1.4×10^6 and 2×10^6 cells per ml, and L-67 cells between 8×10^5 and 10^6 cells per ml. Cells were collected by centrifugation and suspended in medium 67, or the medium indicated in the appropriate experiments, at 10^7 cells per ml, and virus was added to 10 to 30 $TCID_{50}$ per cell (zero-time of infection). The suspension was incubated in a water bath at 50 C for 5 min and for an additional 20 min on a rotary shaker at 37 C. It was then diluted with the appropriate medium (37 C) to 10^6 to 2×10^6 cells per ml and incubated on a rotary shaker at 37 C. Suspensions of uninfected cells were prepared in an identical manner except that the addition of virus was omitted.

Incorporation of labeled amino acids. Suspensions of cells were supplemented with ^{3}H-amino acid mixture as indicated in the appropriate experiments. Duplicate samples of 1 ml were removed at various time intervals, quickly frozen in a bath of solid CO_2 in ethyl alcohol, and subsequently analyzed for radioactivity in acid-insoluble material (21).

Incorporation of labeled uridine. Suspensions of cells were supplemented with FUdR and ^{3}H-uridine as indicated in the appropriate experiments. Duplicate samples of 1 ml were removed at various time intervals, quickly frozen in a bath of solid CO_2 in ethyl alcohol, and subsequently analyzed for radioactivity in acid-insoluble material (21). The incorporation of uridine into virus-specific single-stranded and double-stranded RNA and into virus particles was determined as described elsewhere (20; *see* legend to Fig. 8). The assay is based on the fact that, when infected cells are lysed in solutions of relatively high ionic strength containing 0.5% (w/v) sodium deoxycholate (DOC), single-stranded RNA is readily hydrolyzed by ribonuclease (1 μg/ml) in 30 min at 27 C, whereas double-stranded RNA and RNA in virus are resistant. The RNA in virus, however, is released from the protein capsid of the virus by heating at 80 C for 30 min and is thereby rendered sensitive to degradation by ribonuclease, whereas the ribonuclease resistance of double-stranded RNA is not affected by this treatment.

Zone sedimentation analysis of RNA. About 10^7 labeled cells were harvested from samples of suspension by centrifugation, and the cell pellets were stored at −20 C until analyzed further. Each cell pellet was mixed with 0.3 ml 10% (w/v) sodium dodecyl sulfate (SDS) and 2 ml of 10 mM tris(hydroxymethyl)aminomethane (Tris)chloride (*p*H 7.4). The mixture was incubated at 37 C for 5 min with frequent vigorous agitation and then was layered onto 28 ml of a linear 0.15 to 0.9 M gradient of sucrose in a solution composed of 50 mM NaCl, 10 mM Tris-chloride (*p*H 7.4), 10 mM ethylenediaminetetraacetate (Na_3EDTA), and 0.5% (w/v) SDS. The gradient tubes were centrifuged in a SW25.1 rotor at 18,000 rev/min at 20 C for 15.5 hr.

Sucrose density gradients. Linear sucrose density gradients were prepared by the method of Britten and Roberts (6). Gradients were centrifuged in a Spinco model L-2 ultracentrifuge. After centrifugation, 1-ml fractions were collected by means of a density gradient fractionator (model 180, Instrument Specialties Co., Lincoln, Neb.) which was attached to a continuously recording ultraviolet light analyzer (model UA-2, flow cell of 0.5-cm light path; Instrument Specialties Co.).

Assay for viral RNA polymerase. Cells were collected by centrifugation and suspended in 0.4 ml of B2 (10 mM Tris-chloride, *p*H 7.4; 10 mM NaCl; 1.5 mM $MgCl_2$); the suspensions were stored at −20 C. After 10 to 20 hr, all samples of an experiment were

rapidly thawed at 27 C. Each sample was mixed with 0.05 ml of 10% (w/v) DOC and 0.05 ml of a solution of actinomycin D (200 μg/ml) at 0 C and within 5 min with 0.4 ml of a solution containing (in micromoles): magnesium acetate, 5; Tris-chloride (*p*H 8.2), 50; adenosine, cytidine, and uridine triphosphates (ATP, CTP, and UTP), 0.06 each; ^{3}H-GTP (1,000 μc/μmole), 0.001; sodium phosphoenol pyruvate, 4; and 8 μg of pyruvate kinase. The mixtures were incubated at 27 C for 120 min and then were analyzed for radioactivity in acid-insoluble material as described previously (22).

RESULTS

Effect of viral infection on RNA synthesis by N1S1-67 and L-67 cells. Results from previous experiments (21) in which the technique of pulse-labeling was employed indicated that infection of N1S1-67 cells with mengovirus did not significantly alter the overall rate of uridine incorporation by these cells until the end of the replicative cycle. The results from the experiment presented in Fig. 1a, in which the technique of continuous-labeling was employed, are essentially in agreement with this observation. Infected N1S1-67 cells incorporated uridine into acid-insoluble material at about the same rate as uninfected cells until approximately 1.5 hr after infection. Uridine incorporation by infected cells slowed down slightly between 1.5 and 2 hr but then continued at about the same rate as in uninfected cells until about 5 hr after infection. Subsequent to 2.5 hr after infection, the time course of uridine incorporation was about the same as that exhibited by actinomycin-treated infected cells, which incorporated uridine almost exclusively into virus-specific RNA (21; *see* Fig. 2). The loss of acid-insoluble radioactivity beginning at 5 hr after infection coincided with and probably was a consequence of virus-induced cell degeneration (*see* Fig. 7).

The data in Fig. 1b illustrate that L-67 cells, like the N1S1-67 cells, continued to incorporate uridine at about the same rate as did uninfected control cells during the first 1.5 to 2 hr after infection with mengovirus. It is clear from a comparison of the curves in Fig. 1a and b, however, that uninfected L-67 cells incorporated uridine at only about one-fourth of the rate exhibited by N1S1-67 cells. This difference is probably related to the fact that the growth rate of the L-67 cells is markedly lower than that of the N1S1-67 cells. The rate and extent of synthesis of virus-specific RNA, on the other hand, were almost identical in both strains of cells. The yield of infectious virus was also the same in L-67 and N1S1-67 cells (10,000 HA units per ml of suspension at 7 hr after infection). Results similar to those illustrated in Fig. 1 were obtained in experiments in which the cells of both cell types were suspended in BM42 or Eagle's medium, or in medium 63 instead of medium 67, except that the rate of uridine incorporation by the uninfected cells varied somewhat with the type of medium. In all instances, however, the time course of uridine incorporation by the infected cells was identical to that of the corresponding uninfected cells during the first 1.5 to 2 hr after infection.

The finding that infected cells incorporated uridine between 2.5 and 5 hr after infection at approximately the same rate whether they were treated with actinomycin D or not (Fig. 1) suggested that even in the absence of actinomycin D most of the RNA synthesized by infected cells during this period was virus-specific. Between 2 and 3 hr after infection, therefore, host-directed RNA synthesis appeared to have been replaced by virus-directed synthesis of RNA. The following experiments were conducted to obtain additional information on this point. In a preliminary test, the sedimentation properties of the RNA syn-

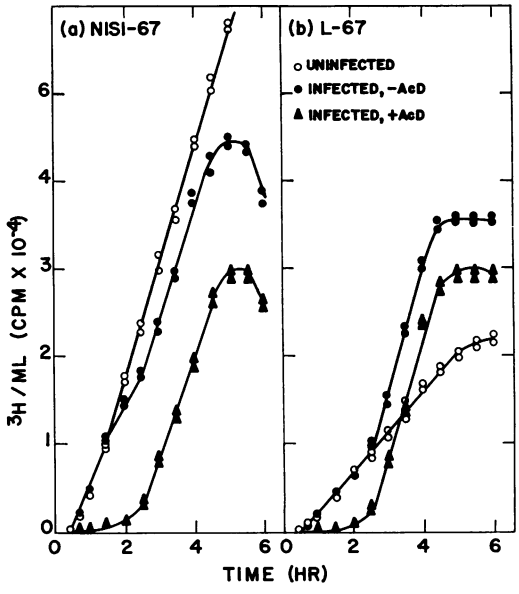

FIG. 1. *Effect of viral infection on RNA synthesis by N1S1-67 (a) and L-67 (b) cells suspended in medium 67. N1S1-67 and L-67 cells were infected with 20 $TCID_{50}$ per cell as described under Materials and Methods (zero-time). Suspensions of infected and uninfected cells in medium 67 at 10^6 cells per ml were supplemented with 0.5 mM FUdR and 5 μM ^{3}H-uridine (100 μc/μmole) at 0.5 hr. Where indicated, 2 μg per ml of actinomycin D (AcD) was added to suspensions of infected cells 5 min prior to FUdR and uridine. Duplicate samples of 1 ml of each suspension were assayed for acid-insoluble radioactivity.*

thesized by actinomycin-treated infected N1S1-67 and L-67 cells were investigated (Fig. 2). Cells were labeled with ^{3}H-uridine between 3.25 and 3.75 hr after infection. A sample of cells from each strain was treated with SDS and then centrifuged through a sucrose density gradient. A second sample of cells was treated with DOC and ribonuclease prior to sedimentation analysis. The data in Fig. 2 show that the virus-specific RNA synthesized by the two strains of cells was similar in both quantity and quality. The RNA which sedimented at 30 to 32*S* was single-stranded viral RNA, as judged by its sensitivity to degradation by ribonuclease, whereas most of the 18*S* RNA was probably double-stranded, because it was resistant to ribonuclease. It was observed that mengovirus RNA, when isolated from infected cells by treatment with SDS, sedimented at about 31*S* as estimated by the method of Martin and Ames (15) with the 29*S* ribosomal RNA (29, 30) as reference standard. In contrast, the RNA isolated from the same strain of mengovirus or from infected cells by extraction with phenol sediments at 34 to 36*S*, whether analyzed in gradients of sucrose in 10 mM Tris-chloride (*p*H 7.4), 10 mM Na_3EDTA (24), or in the sucrose-SDS gradients employed in the present study (not shown). The reasons for this discrepancy are not known. SDS-isolated viral RNA also exhibited a reproducible shoulder on the heavy side of the 31*S* peak (Fig. 2), which was not observed with RNA isolated by phenol extraction.

The following experiment was designed to determine the nature of the RNA synthesized by non-actinomycin-treated, infected cells. Samples of uninfected and infected N1S1-67 and L-67 cells were pulse-labeled for 30 min at various times during a 5-hr incubation period. The RNA was released from the cells by treatment with SDS and was analyzed by centrifugation through sucrose density gradients (Fig. 3). A comparison of the sedimentation profiles presented in Fig. 3 with those in Fig. 2 demonstrates that, even in the absence of actinomycin D, most of the RNA synthesized by infected N1S1-67 or L-67 cells at 3.25 and 4.75 hr after infection was virus-specific. The RNA synthesized by infected cells of either strain between 0.5 and 1 hr after infection, on the other hand, was qualitatively and quantitatively similar to that synthesized by the corresponding uninfected cells. It should be noted that, because of the low incorporation exhibited by uninfected L-67 cells (*see* Fig. 1), the radioactivity profiles in the first two frames of Fig. 3b (L-67 cells) are plotted on a scale one order of magnitude lower than that of the other frames. Label was recovered mainly in 29*S* RNA and a 38*S* RNA which may represent a precursor to ribosomal RNA (14, 18, 26, 28). In agreement with the results of other investigators with various strains of animal cells in culture (13, 14, 26, 28, 31, 34), some of the label incorporated by N1S1-67 and L-67 cells during a 30-min pulse was located in a type of RNA with heterogeneous sedimentation proper-

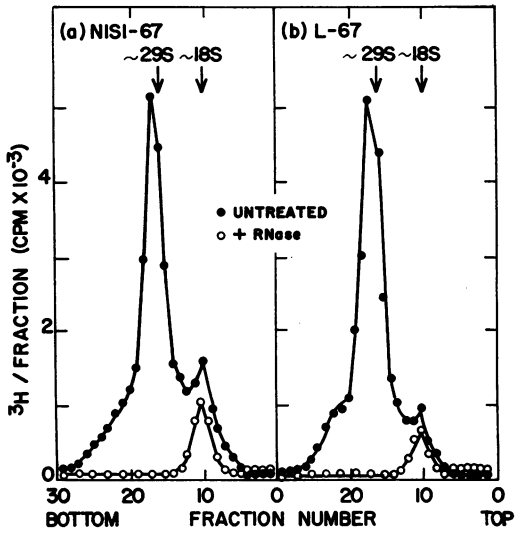

FIG. 2. *Sedimentation analyses of virus-specific RNA produced by N1S1-67 (a) and L-67 (b) cells. At 0.5 hr after infection with 20 $TCID_{50}$ per cell, 2 μg of actinomycin D and 0.5 μmole of FUdR was added per ml of suspensions containing 10^6 N1S1-67 or L-67 cells per ml of medium 67. At 3.25 hr after infection, 0.01 μmole per ml of ^{3}H-uridine (100 μc/μmole) was added, and after 30 min of incubation the cells were harvested from two 10-ml samples of each suspension by centrifugation. The cells of one sample from each suspension were suspended in 2 ml of a solution composed of 0.15 M NaCl, 10 mM Tris-chloride (pH 7.4), 0.5% (w/v) sodium deoxycholate, and 2 μg of ribonuclease per ml and were incubated at 27 C for 10 min. Each suspension was then mixed with 0.3 ml of 10% (w/v) SDS, incubated at 37 C for 5 min, and then analyzed by zone sedimentation. The cells of the remaining samples were suspended in 2 ml 0.15 M NaCl, 10 mM Tris-chloride (pH 7.4) at 0 C, and then were immediately mixed and incubated with SDS as the first set of samples. Conditions of centrifugation: 0.15 to 0.9 M linear gradient of sucrose in 50 mM NaCl, 10 mM Tris-chloride (pH 7.4), 10 mM Na_3EDTA and 0.5% (w/v) SDS; SW25.1 rotor; 18,000 rev/min, 20 C, 15.5 hr. The gradients were monitored for absorbancy at 256 mμ and for acid-insoluble radioactivity (see Materials and Methods). The graphs are composites of the profiles from gradients of untreated and ribonuclease-treated samples. The arrows indicate the positions of the 29S and 18S ribosomal RNA as judged from the absorbancy profiles (see Fig. 3).*

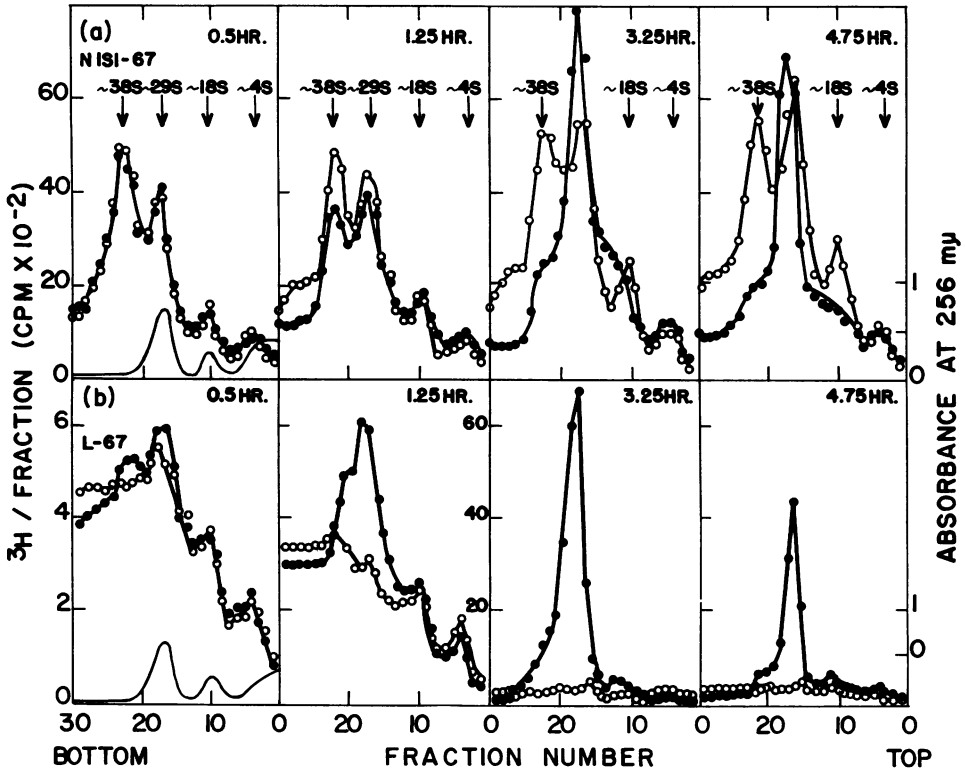

FIG. 3. *Sedimentation analyses of RNA produced by uninfected and infected N1S1-67 (a) and L-67 (b) cells. Suspensions of uninfected and infected cells in medium 67 at 10^6 cells per ml were supplemented with 0.5 mM FUdR at 0.5 hr. At the indicated times, 10-ml samples of each suspension were supplemented with 0.01 μmole per ml of ^{3}H-uridine (100 μc/μmole) and incubated for 30 min. The cells were harvested and treated with SDS, and the resulting suspension was analyzed by gradient centrifugation as described under Materials and Methods. Conditions of centrifugation were as in Fig. 2. The gradients were monitored for absorbancy at 256 mμ (—) and acid-insoluble radioactivity; ○, uninfected cells; ●, infected cells.*

ties, ranging from 20*S* to over 50*S*, and only a small proportion of the total label was present in 4*S* and 18*S* RNA. The shapes of the sedimentation profiles suggest that heterogeneous RNA represented a much greater proportion of the total RNA synthesized by L-67 cells during the 30-min pulse than by N1S1-67 cells. This finding is not unexpected in view of the fact that L-67 cells exhibit a much lower metabolic rate than N1S1-67 cells and that the heterogeneous RNA is the type of RNA synthesized most rapidly by cells (31). The overall profiles of RNA from infected N1S1-67 cells labeled between 1.25 and 1.75 hr after infection (Fig. 3a) was similar to that of uninfected cells, although there was some indication of a somewhat smaller amount of label in some of the RNA molecules. A comparison of the various frames in Fig. 3a shows that, although the synthesis of virus-specific RNA by N1S1-67 cells was accompanied by a marked reduction in the syntheses of various types of cellular RNA, the synthesis of 4*S* RNA appeared to be unaffected by infection. L-67 cells also continued to synthesize 4*S* RNA throughout the replicative cycle (Fig. 3b), but the effect of infection on the synthesis of the other types of cellular RNA was difficult to evaluate because of the relatively large amounts of virus-specific RNA produced by the cells. It is clear, nevertheless, that the synthesis of various types of cellular RNA by L-67 cells was essentially normal at 1.25 to 1.75 hr after infection. During this period, infected cells also appeared to synthesize some RNA sedimenting between 30 and 40*S*, which was not produced by uninfected cells. The possible relationship of this RNA to the infectious process is under further study.

Effect of viral infection on protein synthesis by N1S1-67 and L-67 cells. As indicated by the data in Fig. 4, infection with mengovirus did not

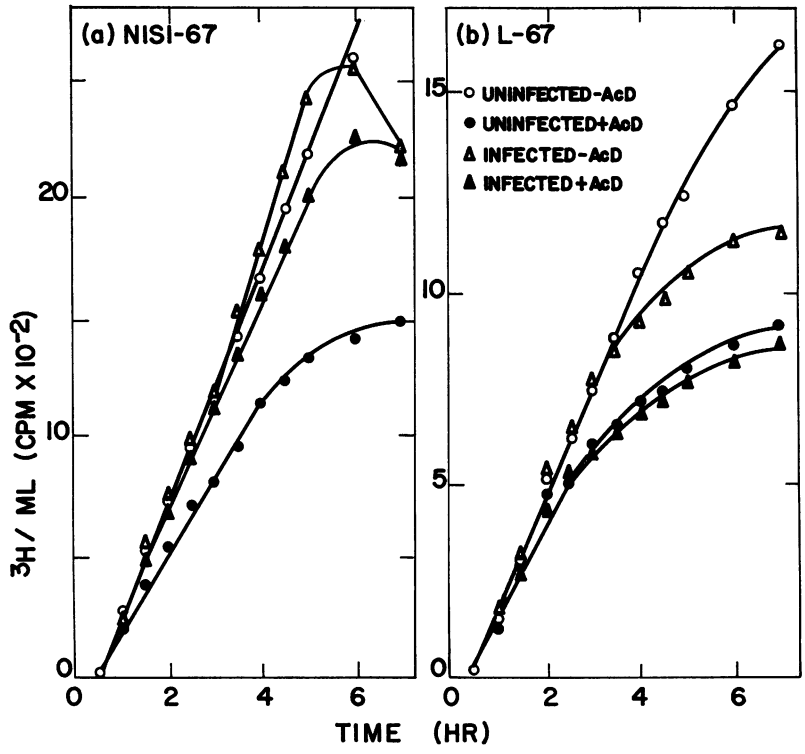

FIG. 4. *Protein synthesis by uninfected and mengovirus-infected N1S1-67 (a) and L-67 (b) cells. Uninfected cells and cells infected with 15 TCID$_{50}$ per cell were suspended in medium 67 at 1.2 × 10^6 cells per ml. At 25 min, a 25-ml sample of each suspension was supplemented with 2 μg of actinomycin D per ml, and another 25-ml sample remained untreated. At 30 min, 1.5 μc of ^{3}H-amino acid mixture (50 to 200 μc/μmole of various amino acids) was added to each flask, and duplicate 1-ml samples were assayed for acid-insoluble radioactivity at various times thereafter. Each point is the average of the duplicate determinations.*

significantly affect the rate of incorporation of amino acids into acid-insoluble material by either N1S1-67 or L-67 cells for the first 2 to 3 hr after infection. Infection resulted in a slight enhancement of amino acid incorporation by N1S1-67 cells between 3 and 5 hr after infection, as was also indicated by results from pulse-labeling experiments (21). The rate of amino acid incorporation by L-67 cells, on the other hand, became depressed during the same period of time. Amino acid incorporation by infected cells was less inhibited by actinomycin D than that of uninfected cells.

That virus infection had little effect on the overall synthesis of proteins by N1S1-67 and L-67 cells is also indicated by the results from sedimentation analyses of cytoplasmic extracts prepared from cells at various times after infection. The profiles illustrated in Fig. 5 show that the polyribosomes of N1S1-67 cells remained intact until late in the replicative cycle. Infection did not result in a rapid disaggregation of the polyribosomes as is observed in HeLa cells after infection with poliovirus (19, 32). On the contrary, most of the 80*S* ribosomes and 115*S* ribosomal structures present at zero-time of infection became associated with large polyribosomal structures during the first 2 hr after infection. This was similar to uninfected control cells incubated under the same conditions (not shown). That most of the cells in the culture were infected was indicated by the fact that close to 100% of the cells became trypan blue-stainable after a single cycle of virus replication (*see* Fig. 7a). Polyribosomal disaggregation became apparent at 5 hr after infection (Fig. 5e) and was almost complete at 6 hr (Fig. 5f). Results similar to those shown in Fig. 5 were obtained with L-67 cells (not shown), except that polyribosome disaggregation became noticeable as early as 4 hr after infection.

Effect of infection on RNA synthesis by L cells derived from monolayer cultures. The results presented so far indicated that the response of L-67 cells to infection with mengovirus is similar to that of N1S1-67 cells, in that the syntheses of

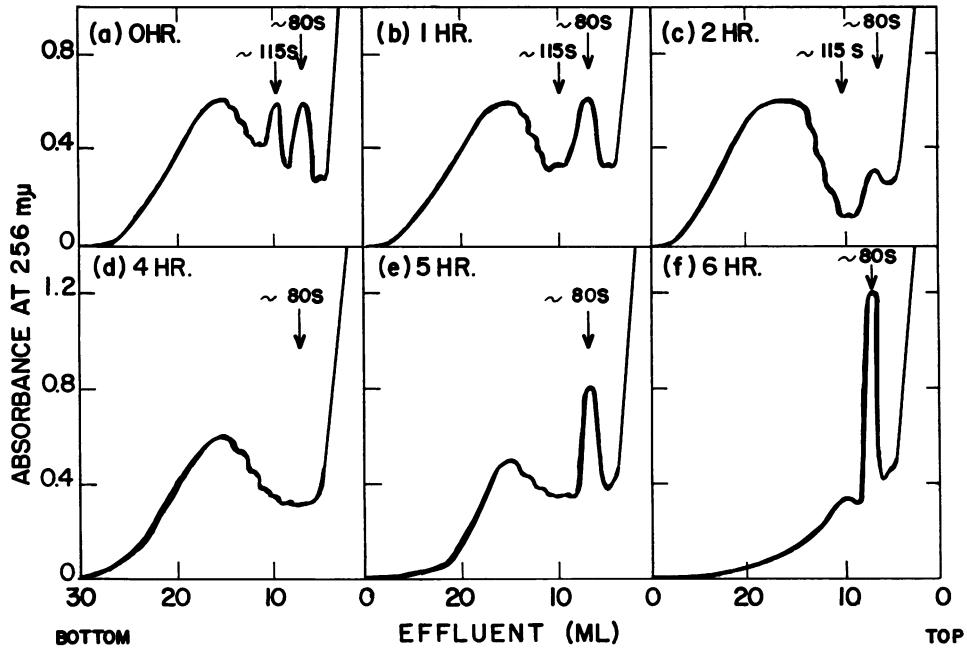

FIG. 5. *Sedimentation analysis of cytoplasmic extracts from virus-infected N1S1-67 cells. Cells (5 × 10^7) were harvested by centrifugation from an exponentially growing culture of N1S1-67 cells (0 hr) and fractionated as described below. Other cells from the culture were infected with 20 $TCID_{50}$ per cell in medium 67 as described under Materials and Methods and samples of 5 × 10^7 cells were harvested at the indicated times. The cells of each sample were suspended in 3 ml of B2, and after 10 min at 0 C were disrupted by 15 strokes with a Dounce homogenizer (21). The nuclei were removed by centrifugation at 1,500 × g for 5 min, and the supernatant fractions were mixed with 0.3 ml of 10% (w/v) DOC at 0 C and then centrifuged through linear 0.5 to 1.2 M gradients of sucrose in B2 in a SW25.1 rotor at 24,500 rev/min at 4 C for 2 hr. The gradients were monitored for absorbancy at 256 mμ (see Materials and Methods).*

cellular RNA and protein are not rapidly inhibited after infection as has been observed by Franklin and his co-workers (4, 10) with their mengovirus-L cell system. To elucidate the question of whether this difference in cell behavior was an intrinsic property of the cells or was due to the use of different strains of mengovirus, it was of interest to determine the effect of infection on RNA synthesis by cells of the original strain of L cells from which the L-67 strain was derived. Cells derived from monolayers were infected with the same strain of mengovirus under conditions identical to those employed in the experiment illustrated in Fig. 1, except that the cells were suspended in Eagle's medium rather than in medium 67. In contrast to the L-67 cells, RNA synthesis by the original strain of L cells was almost completely inhibited at 1 hr after infection (Fig. 6). The time course of synthesis of virus-specific RNA (actinomycin-resistant RNA synthesis), on the other hand, was about the same in both strains of L cells (compare Fig. 1 and 6).

Formation of virus-induced RNA polymerase in N1S1-67 and L-67 cells. Virus-induced RNA polymerase activity appears in mengovirus-infected N1S1-67 cells in a biphasic manner (22, 24; Fig. 7a). An initial increase in activity between 2 and 4 hr after infection is followed by a secondary increase between 5 and 6.5 hr, which coincides with an increase in the number of trypan blue-stainable cells in the culture. Treatment of the cells with 2 μg of actinomycin D per ml for 9 hr prior to infection inhibited the induction of RNA polymerase by the virus, production of virus, and virus-induced cell degeneration (23; Fig. 7a) without interfering with virus adsorption (23). Results from analogous experiments with L-67 cells (Fig. 7b) showed that viral RNA polymerase began to appear in these cells at about the same time after infection with mengovirus as in N1S1-67 cells and increased to a similar extent between 2 and 4 hr. At 4 hr after infection, the specific activity (counts per minute incorporated per 8 × 10^6 cells) was about the same in both cell strains. In L-67 cells, however, the activity rapidly disappeared between 4 and 6 hr, and there was only a

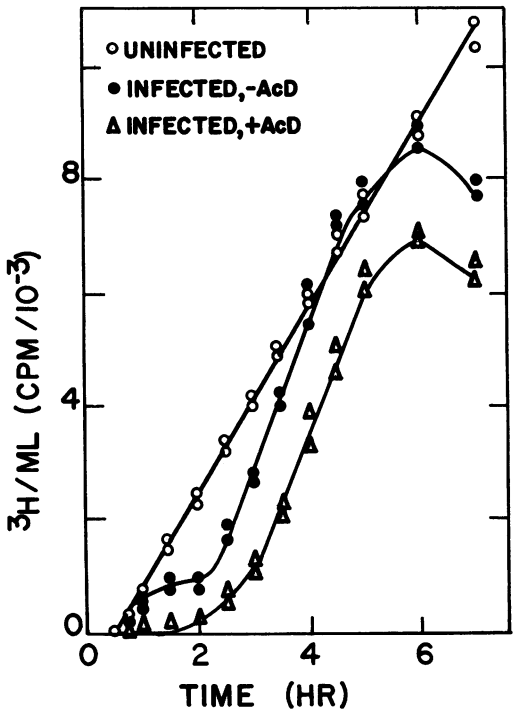

FIG. 6. *Effect of viral infection on RNA synthesis by L cells derived from monolayers. Cells were harvested from monolayer cultures by scraping with a rubber policeman, and a portion of the cells was infected with 20 $TCID_{50}$ per cell as described under Materials and Methods. Suspensions of infected and uninfected cells at 5 × 10^5 cells per ml of Eagle's medium were supplemented with 0.5 mM FUdR and 4 μM ^{3}H-uridine (100 μc/μmole) at 0.5 hr. Actinomycin D (2 μg/ml) was added to one suspension of infected cells 5 min prior to FUdR and uridine. Samples of 1 ml of each suspension were analyzed for acid-insoluble radioactivity.*

slight secondary increase coincidental with an increase in the number of trypan blue-stainable cells in the culture. It is of interest that virus-induced cell degeneration, as measured by vital staining with trypan blue, commenced about 2 hr later in L-67 cells than in N1S1-67 cells. Treatment of the L-67 cells with 2 μg of actinomycin per ml 9 hr prior to infection did not affect the initial increase in polymerase activity significantly (Fig. 7b) and had no effect on virus yield, as judged from HA assays (not shown), but the secondary increase in activity and virus-induced cell degeneration was markedly reduced. Actinomycin D at 2 μg per ml inhibited the incorporation of uridine into acid-insoluble material about 94% in L-67 cells and over 96% in N1S1-67 cells within 15 min of its addition. Results from other experiments (not shown) demonstrated that pretreatment of L-67 cells with 10 μg of actinomycin D per ml had as little effect on the induction of RNA polymerase activity and virus production as the treatment with 2 μg per ml, but further reduced the trypan blue stainable cells present in the culture at 12 hr after infection to less than 5%.

Time course of synthesis of virus-specific single-stranded and double-stranded RNA by L-67 cells and incorporation of RNA into virus. The synthesis of single-stranded and double-stranded RNA by infected L-67 cells and the incorporation of RNA into virus was determined by the same procedure previously (20, 21) employed in studying the various processes in mengovirus-infected N1S1-67 cells (*see* Materials and Methods). The results presented in Fig. 8 show that the synthesis of single-stranded and double-stranded RNA by infected L-67 cells followed a similar time course throughout the replicative cycle and that the beginning of the synthesis of the two types of RNA preceded the incorporation of RNA into virus by about 1 hr. About 8% of the total RNA synthesized by the cells in the presence of actinomycin D was double-stranded and the remainder was single-stranded (*see* Fig. 2), and only about 18% of the total RNA was incorporated into virus. These results are very similar to those obtained with mengovirus-infected N1S1-67 cells (20, 21). In L-67 cells, as in N1S1-67 cells, virus maturation was confined to the period between 4 and 6 hr after infection, as indicated by both the biochemical determinations and the HA assays (Fig. 8). As indicated by the HA determinations, however, the release of virus from the cells was very slow and was only beginning at about 7 hr after infection. A comparison of the data in Fig. 8 and 7b indicates that virus release coincided with an increase in the number of trypan blue-stainable cells in the culture. In contrast, mature virus is released from infected N1S1-67 cells very rapidly and efficiently after the cessation of virus maturation (21) but also occurs coincidentally with the degeneration of the cells (*see* Fig. 7a).

DISCUSSION

It is unlikely that the failure to observe an inhibition of RNA and protein synthesis and a loss of polyribosomes in N1S1-67 or L-67 cells during the early stages of infection with mengovirus is simply due to a lack of synchrony of the infective process in the population or the presence of large numbers of uninfected cells. On the contrary, the fact that the entire production of progeny virus in these cultures occurs within a 1.5- to 2-hr period (Fig. 8) and that close to 100% of the cells in infected cultures become stainable

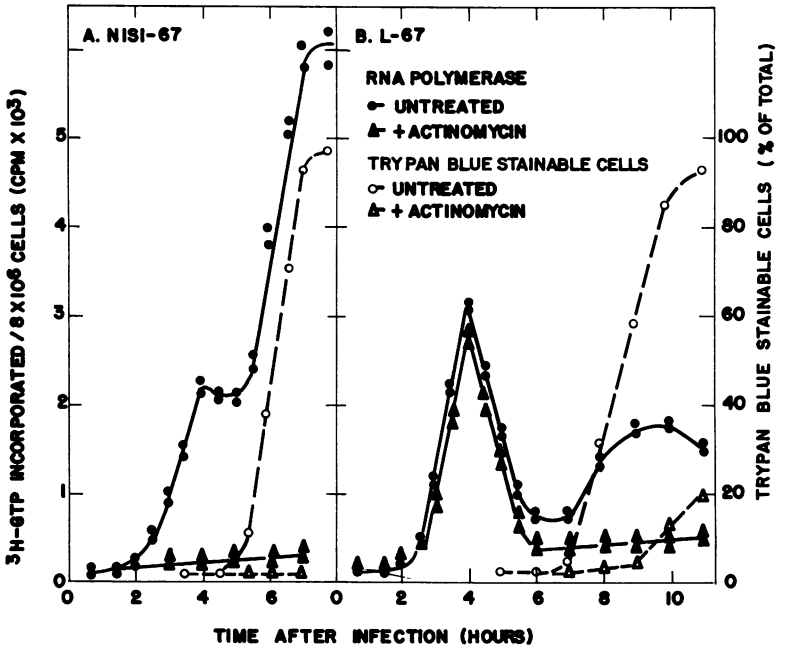

FIG. 7. *Time courses of formation of virus-induced RNA polymerase and of virus-induced cell degeneration in actinomycin-treated and untreated N1S1-67 (a) and L-67 (b) cells. Portions of cultures of exponentially growing N1S1-67 and L-67 cells were supplemented with 2 μg of actinomycin D per ml. Other portions of these cultures remained untreated. After 9 hr of incubation, the cells of each suspension were harvested, infected with 25 TCID$_{50}$ per cell, and finally suspended in BM42 at 2 × 10^6 cells per ml. The cells from 4-ml samples were analyzed for RNA polymerase activity, and cells from other samples, for stainability by trypan blue, as described under Materials and Methods.*

by trypan blue within a similar period of time (Fig. 7) suggests that, under the conditions of the experiments, virus replication is highly synchronized and that almost all of the cells are infected. N1S1-67 and L-67 cells, therefore, appear to differ from many other strains of animal cells, the macromolecular synthesis of which is rapidly inhibited by infection with a number of picornaviruses (4, 11, 16, 35). Cellular RNA and protein synthesis by HeLa cells is similarly unaffected for several hours after infection with mengovirus (17). That the relative resistance of RNA and protein synthesis by N1S1-67 and L-67 cells to the inhibitory effect of mengovirus is an intrinsic property of the cells rather than of the strain of mengovirus or due to the experimental conditions is indicated by the findings that (i) RNA synthesis by cells of either strain continues at a normal rate whether the cells are suspended during or after infection in BM42, medium 67, medium 63, or Eagle's medium, or whether the infected cells are incubated at 35 or 37 C (*unpublished*); and (ii) RNA synthesis by cells of the original strain of L cells (Fig. 6) and by N1S1-63 cells (21; *unpublished*) is rapidly inhibited by infection with the same strain of mengovirus under otherwise comparable experimental conditions. The molecular basis for the relative resistance of N1S1-67 and L-67 cells to the inhibitory effect of mengovirus is not known, but it seems likely that the composition of medium 67 plays an important role in the selection of mutant cells which do not respond to viral infection with a rapid cessation of cellular RNA and protein synthesis. Whether this selective pressure is due to the relatively low serum content (5% calf serum) of medium 67, the presence of the pancreatic autolysate, or other factors is not clear. Results from additional studies (*unpublished*) have shown that N1S1-67 cells again became sensitive to the inhibitory effects of mengovirus during growth for 20 passages in medium 63, which has the same basic composition as medium 67 but lacks the pancreatic autolysate and contains 15% horse serum instead of 5% calf serum. The altered response of these cells to viral infection was probably a characteristic of a nutritional mutant of N1S1-67 cells which was favored in medium 63 and developed during passage, rather than a phenotypic characteristic of cells grown in

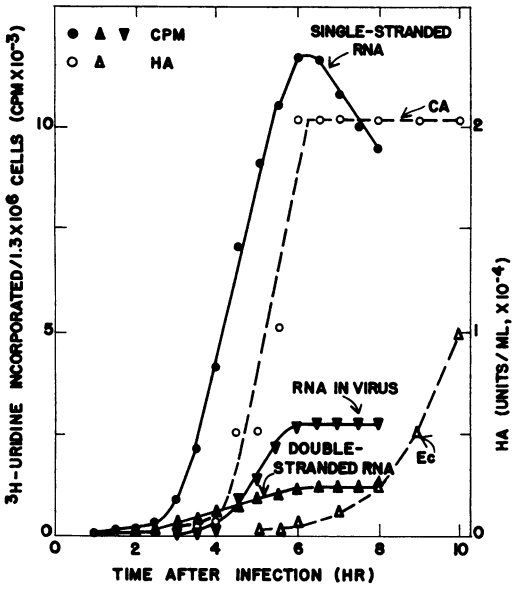

FIG. 8. *Time courses of syntheses by L-67 cells of virus-specific single-stranded and double-stranded RNA, the incorporation of RNA into virus, and the formation of cell-associated and extracellular virus. A suspension of infected L-67 cells in BM42 at 1.3 × 10^6 cells per ml was supplemented with 2 μg of actinomycin D per ml and 0.5 mM FUdR at 25 min after infection; 5 min later, 5 μM ^{3}H-uridine (100 μc/μmole) was added. At various time intervals thereafter, six replicate 0.5-ml samples were rapidly frozen in a bath of solid CO_2 in ethyl alcohol. These samples were later thawed and diluted with 1.5 ml of 0.15 M NaCl, 10 mM Tris-chloride (pH 7.4), and duplicate samples were treated as follows: (1) stored at 0 C until (2) and (3) were ready for analysis; (2) 0.05 ml of 10% (w/v) DOC and 0.05 ml of a solution of ribonuclease (50 μg/ml) were added and the mixture was incubated at 27 C for 30 min; and (3) samples were heated at 80 C for 10 min, cooled, and treated with DOC plus ribonuclease as described under (2). All samples were analyzed for acid-insoluble radioactivity. The amount of radioactivity in free single-stranded RNA, double-stranded RNA, and in virus was calculated as follows (20): free single-stranded RNA (●) = radioactivity in (1) minus that in (2); RNA in virus (▼) = (2) minus (3); and double-stranded RNA (▲) = (3). Each point represents the average of duplicate determinations. Other 1-ml samples were centrifuged and the supernatant fluids were assayed for extracellular (EC) HA activity. Each pellet was suspended in 1 ml of BM42 containing 1% DOC and then assayed for cell-associated (CA) HA activity.*

medium 63, since after the fifth passage of the N1S1-67 cells in medium 63 the cells still behaved identically to the original N1S1-67 cells.

Although cellular RNA synthesis continues normally in N1S1-67 and L-67 cells for the first 2 hr after infection, it is rapidly displaced by the synthesis of virus-specific RNA between 2 and 3 hr (Fig. 3). Only the incorporation of uridine into 4*S* RNA continues normally throughout the replicative cycle. Whether the latter represents de novo synthesis of 4*S* RNA or mainly end-group labeling has not been determined. The mechanism by which the transition from cell RNA synthesis to viral RNA synthesis is induced is not understood, nor is it known whether the same processes are involved as in the rapid inhibition of RNA synthesis by virus infection in other strains of cells. The transition from cellular protein synthesis to the synthesis of virus-specific proteins also must occur very smoothly, since the rate of incorporation of amino acids continues at the normal rate for at least 3 hr after infection (Fig. 4) and there is no detectable disaggregation of polyribosomes until late in the replicative cycle (Fig. 5). It is not clear how viral RNA replaces cellular messenger RNA on the polyribosomes, but the results indicate that under the experimental conditions employed in this study the cells at 2 hr after infection do not possess a large pool of unused 80*S* ribosomes or of ribosomal subunits to which newly synthesized viral RNA could attach. Viral protein, however, appears to represent only a small proportion of the total protein synthesized by infected N1S1-67 and L-67 cells. Throughout the replicative cycle, the bulk of the protein synthesized probably continues to be cellular protein. This is suggested by the observation that the rate of protein synthesis in infected cells is not related to the amount of virus produced. The rate of amino acid incorporation by infected L-67 cells, for instance, is appreciably lower and is inhibited to a greater extent by infection than is that by N1S1-67 cells Fig. 4), in spite of the fact that the amount of virus produced by the two cell lines is about the same. The failure to detect in mengovirus-infected N1S1-67 or L-67 cells the formation of large (400*S*) virus-specific polyribosomes (Fig. 5), as has been observed in poliovirus-infected HeLa cells (19), may be due to the continued presence of a large number of normal cellular polyribosomes.

The time courses of synthesis of virus-specific single-stranded and double-stranded RNA and of the incorporation of RNA into virus are very similar in N1S1-67 and L-67 cells (Fig. 1 and 8; 20, 21), and similar amounts of viral RNA and virus are produced when the cells are infected under identical experimental conditions and with the same strain of mengovirus. These observations are of interest because they suggest that under optimal conditions the time course of viral replication is largely determined by the viral

genome rather than by the metabolic rate of the host cell. The cells, however, appear to possess a pool of nucleotide triphosphates sufficiently large to saturate the RNA polymerases, and the rate of RNA synthesis by infected cells appears to be mainly a reflection of the catalytic properties of the virus-induced RNA polymerase. Thus, infection of L-67 cells results in a marked stimulation of overall RNA synthesis, whereas the rate of RNA synthesis by N1S1-67 cells remains relatively constant after infection (Fig. 1).

In both types of cells, the initiation of synthesis of virus-specific RNA coincides with the appearance of viral RNA polymerase activity, as measured in cell-free extracts (Fig. 7). Most of the RNA polymerase which is produced in infected L-67 cells between 2 and 4 hr after infection, however, appears to be metabolically unstable and disappears subsequently. A similar apparent instability of the viral RNA polymerase is observed in cells infected with poliovirus (2), EMC virus (12), and foot-and-mouth disease virus (25). A comparison of the data in Fig. 7b and 8 clearly indicates that polymerase activity begins to decrease coincidentally with the commencement of viral maturation and that most of the enzyme activity has disappeared when maturation ceases. When infected N1S1-67 cells are treated with puromycin, the viral RNA polymerase activity also decays very rapidly, and the apparent instability of the enzyme is also confined to the period of viral maturation (22). A loss of polymerase activity may also be associated with viral maturation in N1S1-67 cells, but this may be obscured by the marked secondary increase in polymerase activity which accompanies the virus-induced degeneration of these cells between 5 and 7 hr after infection. The decrease of polymerase activity in EMC virus-infected cells also occurs during the period of virus production (12). This close temporal relationship between the metabolic instability of the virus-induced RNA polymerase and virus maturation is probably more than coincidental, but its nature is not understood (*see* 24). The secondary increase in polymerase activity which accompanies cell degeneration probably is due to an activation of template rather than de novo synthesis of polymerase, since it occurs in cells in which protein synthesis is inhibited over 90% by treatment with phenethyl alcohol (20).

Although the time course and extent of mengovirus production are similar in the two types of cells, the results indicate that the virus is released from infected L-67 much slower and less efficiently than from NlSl-67 cells, and this is correlated with a much slower rate of degeneration of these cells (Fig. 7 and 8). Thus, the rate of virus-induced cell degeneration is not only a characteristic of the replicating virus (1, 8), but it can be markedly modified by the host cell. Since cell degeneration may be a consequence of a virus-induced release of hydrolytic enzymes from lysosomes (9, 33), it seems that this process may be more readily activated by the virus in L-67 than in N1S1-67 cells. The finding that the 9-hr pretreatment with actinomycin D markedly reduces the virus-induced cell degeneration of L-67 cells without interfering with viral replication per se (Fig. 7) suggests that either the hydrolytic enzymes of the cells decay or some other cellular factor required for the activation of cell degeneration is lost in the presence of actinomycin D. This appears to be a slow decay, since addition of actinomycin D to cells at the time of infection does not significantly alter the time course or extent of cell degeneration (*unpublished*).

The results reported here also confirm the previous findings (10, 23) which indicated that treatment of L cells for prolonged periods of time with actinomycin D does not affect the subsequent replication of mengovirus, whereas viral replication is completely inhibited by Novikoff cells under identical conditions. Since RNA synthesis by N1S1-67 and L-67 cells is inhibited by actinomycin D to about the same degree, the difference in sensitivity of viral replication to actinomycin D in these two types of cells does not appear to be due to a difference in the response of these cells to the action of the antibiotic per se. It seems more likely that Novikoff cells become resistant to virus infection because an unstable cellular component required for viral replication decays during treatment with actinomycin D, whereas this component is presumably more stable in L cells. Whether this may be partly related to the more rapid growth rate of the Novikoff cells is not clear. The requirement of an unstable cellular component for the replication of poliovirus in a number of strains of human cells has also been postulated by Cooper (7), because actinomycin D inhibits viral replication when the cells have been grown in media of low serum content or are in the stationary phase of growth prior to infection. Varying amounts of the hypothetical cell component appear to be required for the replication of different strains of poliovirus (7, 27). The differences in response of the N1S1-67 and L-67 cells to infection with mengovirus, however, are clearly characteristics of the cells, since in the present experiments the cells were infected with the same strain of virus and under identical experimental conditions. A further comparison of the replication of mengovirus in these two types of cells may contribute additional

information with respect to the involvement of host-cell factors in viral replication and viral cytopathology.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI 07250-02.

Technical assistance was provided by John Erbe.

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