*NONPERMISSIVE INFECTIONS OF MAMMALIAN CELLS: SYNTHESIS OF INFLUENZA VIRUS GENOME IN HeLa CELLS**

By R. A. Lerner and L. D. Hodge†

WISTAR INSTITUTE, PHILADELPHIA, PENNSYLVANIA

Communicated by Karl Habel, July 25, 1969

Abstract.—The RNA synthesis of a single-stranded, multisegmented viral genome has been investigated in nonpermissive host cells. In HeLa cells, both single- and double-stranded influenza viral RNA were made and accumulated, and synthesis was completed by 15 hours. The evidence indicated that all pieces of the genome were synthesized. At least as much viral RNA was synthesized in HeLa cells as in permissive chick embryo fibroblasts cells. Explanations for this nonpermissive infection based on reduced synthesis of viral RNA or rapid destruction of newly synthesized RNA, uncontrolled synthesis of RNA, or partial synthesis of the genome appear to have been ruled out by this investigation. The persistently high amounts of double-stranded RNA, combined with the reported lack of dispersion of nucleocapsid protein from the nuclear region of the HeLa cell are consistent with a failure of assembly of the virion in this host. This nonpermissive infection of HeLa cells with influenza virus may offer a unique system for the accumulation of the intermediates involved in the assembly of a complex RNA viral genome.

The nonpermissive infection of HeLa cells with influenza virus appears to be an interesting system for observing the synthesis and assembly of a viral genome composed of single-stranded RNA segments. In HeLa cells virus-specific proteins are synthesized, as can be detected by the techniques of hemadsorption and fluorescent microscopy. Virtually every HeLa cell can be infected and no infectious virus is formed, regardless of the multiplicity of infection.[1, 2] This failure to form infectious virus differs from the von Magnus phenomenon seen in chick embryo fibroblasts because the latter is related to high multiplicities of infection. Furthermore, unlike the permissive infection of chick embryo fibroblasts cells, the viral nucleocapsid protein remains in the nuclear region in the HeLa cell infected with influenza and other myxoviruses.[3–6]

In this paper some aspects of the synthesis of influenza viral RNA in HeLa cells are described. The data demonstrate that viral RNA is synthesized and accumulates, and that synthesis is complete 15 hour post infection. Approximately 30 per cent of this viral RNA is resistant to digestion by RNase and all pieces of the genome appear to be synthesized.

Materials and Methods.—Preparation of virus: The PR-8 strain of influenza virus was propagated according to standard methods.

Maintenance and infection of cells: Uncloned HeLa cells or chicken embryo fibroblasts cells were maintained in monolayer culture in Eagle's monolayer medium[7] supplemented with 5% fetal calf serum. Primary chick embryo fibroblast cells were prepared from 9-day-old embryonated eggs and maintained at 37° in 5% CO_2 in the same medium. Confluent monolayers containing approximately 1–4 $\times$ 10^7 cells were used for infection. Monolayers were washed with prewarmed (37°) phosphate-buffered saline and overlaid

with crude allantoic fluid containing 100 ID_{50} units of virus per cell. After absorption at 37° for 60 min, virus was removed, monolayers were washed with phosphate-buffered saline, and 20–50 ml of fresh, complete medium prewarmed at 37° were added. Hemadsorption assays showed that greater than 90% of the cells were infected. (Calculation of post infection time is the time of the replacement of the medium.)

Labeling and extraction of RNA: Infected HeLa cells were resuspended in fresh prewarmed (37°) medium at 2×10^6 cells/ml. Since chicken embryo fibroblast cells did not tolerate this procedure, they were used in monolayers and compared to HeLa cell monolayers containing the same number of cells. Cellular RNA synthesis was inhibited by adding 2.0 μg/ml of actinomycin D 60 min prior to the addition of uridine-^{14}C (30 mc/m mole, New England Nuclear Corp., Boston, Mass.). This concentration of actinomycin D inhibited 95% of the incorporation of ^{14}C-uridine into host-cell RNA. The residual incorporation of isotope has been shown to be into transfer RNA.[8, 9] The time of addition of actinomycin D bypassed the period when influenza viral RNA synthesis is sensitive to this inhibitor.[10] Cells were chilled to 5°, washed twice in cold Earle's saline, and lysed by resuspension in STE (0.1 *M* NaCl, 0.05 *M* Tris-HCl pH 7.4, 0.001 *M* EDTA) containing 1% SDS and 1% mercaptoethanol. After 5 min, the cellular lysate was shaken with an equal amount of redistilled 80% phenol containing 0.1% of 8-hydroxyquinolin at room temperature (22°) for 5 min. The phenol extraction was repeated twice, the aqueous phase was removed, and RNA was precipitated with 3 volumes of 95% ethanol and 0.1 volume of 2 *M* NaCl at −20° for 16 hr. The precipitate was washed twice in ethanol, and resuspended in 1.0 ml of LTM (0.14 *M* LiCl, 0.01 ethanol *M* Tris-HCl pH 7.4, 0.001 *M* $MgCl_2$). This solution was treated with 100 μg DNase (electrophoretically purified, RNase-free deoxyribonuclease I, Worthington, Freehold, N. J.) for 30 min at room temperature and RNA was reprecipitated.

Sedimentation analysis of RNA: RNA labeled with radioactive uridine was resuspended in 1.0 ml STE containing 1% SDS, 1% mercaptoethanol, and separated in 15 to 30% (w/w) linear sucrose gradients containing 0.5% SDS, 0.01 *M* Tris-HCl pH 7.2, 0.1 *M* NaCl, and 0.001 *M* EDTA. Gradients were centrifuged at 24,000 rpm for 17 hr at 20° in a Spinco SW 25.1 rotor and analyzed for absorbance at 260 mμ using a continuous flow cell and a Gilford spectrophotometer. Approximately 0.8 ml fractions were collected. After the addition of carrier (15 μg) of yeast nucleic acid the samples were adjusted to a final concentration of 12.5% with trichloroacetic acid (TCA) and the resulting precipitates were collected onto Millipore filters. The amount of radioactivity was determined. *S* values were determined according to the method of Martin and Ames[11] utilizing 28*S* and 18*S* ribosomal RNA as sedimentation markers.

Preparation of double-stranded RNA: Samples of RNA were resuspended in 1.0 ml of prewarmed (37°) 2 × SSC (0.15 *M* NaCl, 0.015 *M* sodium citrate) containing 2 μg of RNase, and the reaction mixture was incubated at 37° for 30 min. This treatment digested the cellular 28*S* and 18*S* RNA leaving a single 11*S* species of radioactive RNA. Prior to separation in 2.1% agarose-acrylamide gels according to the method of Watanabe *et al.*,[12] residual RNase was removed by extraction 3 times with phenol in the presence of 2 mg of bovine serum albumin (Armour 3 × crystallized).

To prepare double-stranded RNA by salt fractionation, the sample was dissolved in 0.5 ml of STE which was made 2 *M* with respect to NaCl and chilled at 4° for 24 to 48 hr. After centrifugation at 20,000 *g* for 30 min at 4°, the RNA in the supernatant fraction was precipitated and separated in 2.1% agarose-acrylamide gels.

Results.—Synthesis and accumulation of viral RNA: Since no information was available concerning viral RNA synthesis in HeLa cells, initial experiments were to determine if viral RNA was synthesized. Virus-specific RNA was synthesized $3^1/_2$–7 hour post infection. Furthermore, the amount of radioactive uridine incorporated per cell into viral RNA between 3 and 6 hour post infection was similar for chick embryo fibroblasts and HeLa cells. To define the species of viral RNA synthesized, radioactive viral RNA was analyzed by sedimenta-

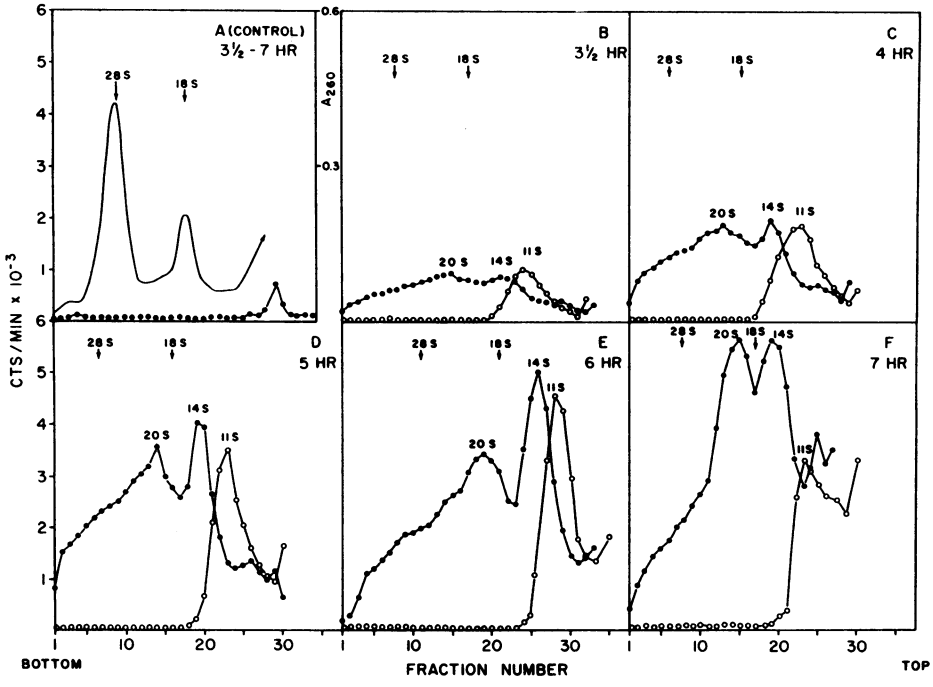

FIG. 1.—Velocity sedimentation of viral RNA. Approximately 4 × 10^8 cells were infected and resuspended 2½ hr later in medium containing actinomycin D. Following 1 hr incubation with inhibitor, 0.5 μc/ml of uridine-2-^{14}C was added. Equal aliquots were removed at the time (post infection) indicated and RNA extracted. Each RNA sample was divided in two: one was treated with 2 μg/ml of RNase before separation; the second was separated in 15 to 30% SDS gradients without enzymatic treatment. The amount of radioactive RNA in each gradient was normalized on the basis of the amount of 28*S* and 18*S* cellular RNA present in the sample not treated with RNase.

Panel *A:* shows the results obtained with control non-infected cells treated with inhibitor and exposed to uridine-2-^{14}C 3½ to 7 hr post mock-infection. For simplicity, the optical density profile of cellular 28*S* and 18*S* has been included only in Panel A.

Panels *B–F:* ●—● total viral RNA; O—O RNase-resistant viral RNA.

tion in sucrose velocity gradients. The profile of sedimentation showed 14 and 20*S* peaks as well as RNA sedimenting faster than 28*S* (Fig. 1, *B–F*). This dispersed sedimentation of viral RNA was significant since 28*S* and 18*S* cellular RNA sedimented as discrete species (Fig. 1*A*) and the conditions of sedimentation minimized aggregation of RNA. The change in profiles during infection indicated that RNA in the 14*S* and 20*S* region of the gradient was accumulating.

When the RNA samples were treated with RNase (2 μg/ml) prior to sedimentation, a single broad peak of radioactivity at approximately 11*S* was obtained (Fig. 1, *B–F*). This 11*S* RNA was 28–36 per cent of the total viral RNA synthesized (Fig. 3 *insert*) and was not present in samples untreated prior to sedimentation. This indicated that the RNA which in undigested samples sedimented throughout the gradient contributed to this RNase-resistant RNA. To confirm this, undigested RNA was separated in sucrose gradients and the individual

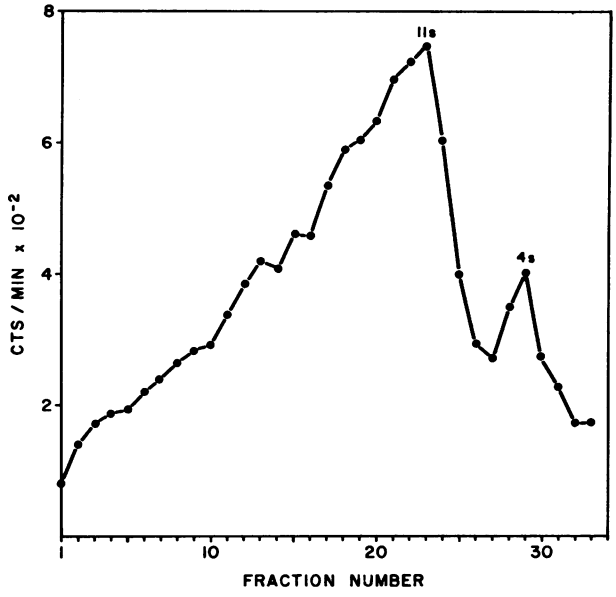

FIG. 2.—Velocity sedimentation of RNase-resistant RNA. Approximately 4×10^8 cells were infected, resuspended, treated with actinomysin D, as described in Fig. 1, and exposed to 0.5 μc/ml of uridine-2-^{14}C from 3 to 6 hr post infection. The extracted radioactive viral RNA was separated in 15 to 30% SDS sucrose gradients. The condition of sedimentation and collection of fractions were the same as described in Fig. 1. The RNA in each fraction was precipitated, washed with 95% ethanol, digested with 2 μg of RNase, and the TCA-insoluble radioactivity determined.

fractions were digested with RNase prior to precipitation with TCA and determination of radioactivity. The 11*S* peak of RNA, with a broad tail extending into the denser region of the gradient (Fig. 2), was typical of the profile described for intermediates involved in the replication of viral RNA. Less than 6 per cent of the RNA in the 14*S* and 20*S* regions of the gradient was resistant to digestion; this suggests that the 14*S* and 20*S* RNA species are predominately single-stranded.

Kinetics of viral RNA synthesis: The analysis of the total RNA and RNase-resistant RNA which accumulated with time (Fig. 3) suggested that the net synthesis of viral RNA was decreasing between six and seven hours after infection. To eliminate the possibility that this cessation represented a depletion of radioactive uridine in the medium and to determine the types of RNA made at different times, infected cells were exposed for 30-minute intervals to ^{14}C-uridine between 3 and 15 hours post infection. The rate of viral RNA synthesis reached a maximum four hours post infection and gradually decreased over the next 11 hours (Fig. 4). The profiles of the RNA and RNase-resistant RNA which were synthesized at various times during infection were similar. The pronounced 14*S* and 20*S* RNA which accumulate during infection (Fig. 1) were noted here (Fig. 5). Approximately 30 per cent of the viral-specific RNA synthesized at any time was resistant to enzymatic digestion with RNase (Fig. 4, *insert*) and sedimented at the 11*S* region of the sucrose gradient (Fig. 5).

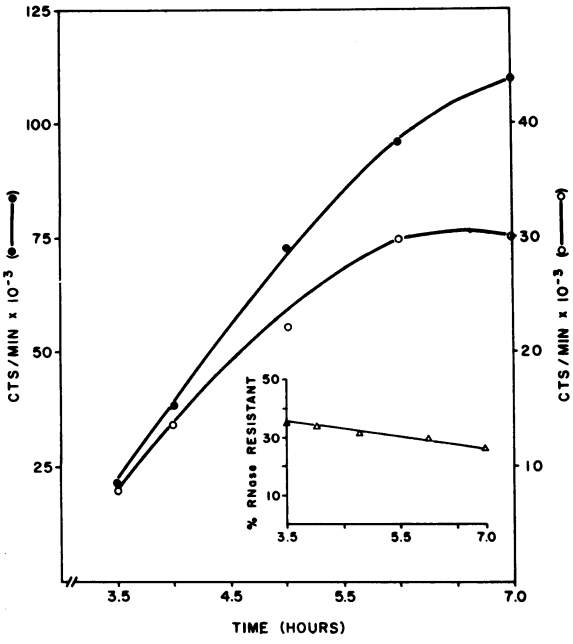

FIG. 3.—Kinetics of accumulation of viral RNA and RNase-resistant RNA. The data are derived from the experiment detailed in Fig. 1 and each point represents the sum of the total TCA-insoluble radioactivity from each sucrose gradient. *Insert:* shows percent of the RNA which was resistant to RNase at the times indicated. ●—● total viral RNA; O—O RNase-resistant viral RNA; △—△ % RNase-resistant.

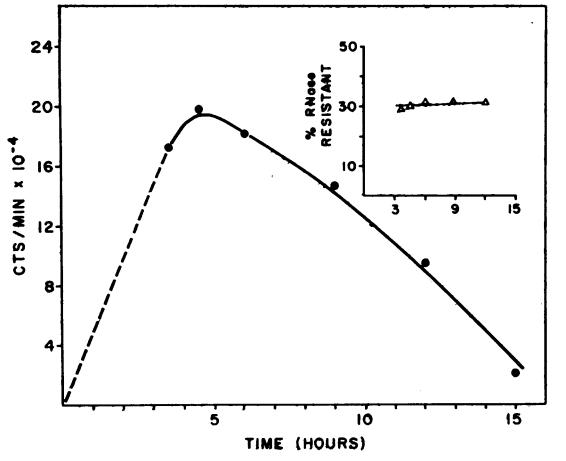

FIG. 4.—Rate of synthesis of viral RNA and RNase-resistant RNA. The data are derived from the experiments detailed in Fig. 5 and each point represents the sum of the total TCA-insoluble radioactivity from each sucrose gradient. The dashed line represents the presumed time course of viral RNA synthesis during the actinomycin D sensitive period of viral RNA replication. *Insert:* shows percentage of the RNA which was resistant to RNase at the times indicated. ●—● total viral RNA; △—△ % RNase-resistant.

Evidence for the synthesis of multiple species of RNA: Because the influenza genome consists of multiple species of single-stranded RNA, it was important to determine if all pieces of the genome were synthesized in the nonpermissive host. Since adequate separation of newly synthesized double-stranded RNA could be achieved by acrylamide gel electrophoresis, the double-stranded RNA patterns were used to obtain evidence for complete genomic synthesis. Viral RNA synthesized between three and six hours post infection was divided into two equal portions. One of these was treated with RNase and again 30 per cent of the viral-specific RNA was found to be resistant to digestion. The other portion was made 2 *M* with respect to NaCl, and after precipitation, the supernatant

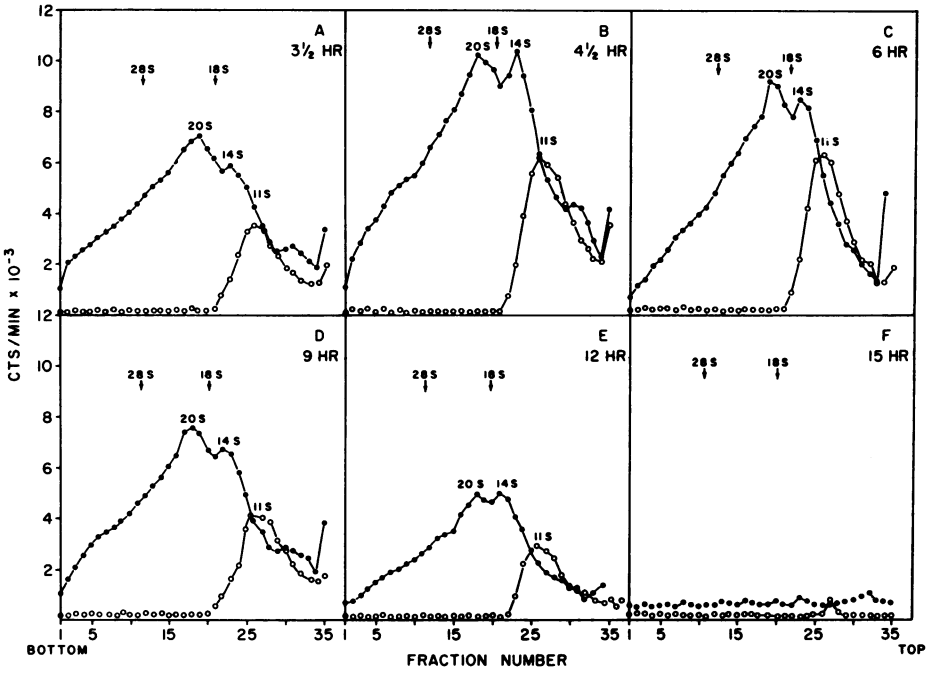

FIG. 5.—Velocity sedimentation of pulse-labeled viral RNA. Approximately 4×10^8 cells were infected and resuspended $2^1/_2$ hr later in medium. One hour prior to the indicated times aliquots were removed, treated with actinomycin D and then pulse-labeled with uridine-2-^{14}C (0.5 μc/ml) for 30 min. RNA was extracted, divided into two aliquots, and the untreated RNA and RNase-resistant RNA were separated in sucrose gradients. The amount of radioactive RNA in each gradient was normalized as described in Figure 1. ●—● total viral RNA; O—O RNase-resistant viral RNA.

fraction contained 7 per cent of the total viral RNA, presumably representing free double strands (RF). Acrylamide gel electrophoresis resolved the salt-soluble and enzyme-resistant RNA into seven species (Fig. 6*A* and 6*B*) with similar distances of migration. The similarity between these profiles eliminates the possibility that the seven double-stranded pieces of RNA might be an artifact of *in vitro* digestion with RNase. The most rapidly migrating polydisperse RNA, seen in Fig. 6*A* but not in Fig. 6*B*, indicates, however, that some fragmentation by RNase does, in fact, occur.

Discussion.—Influenza viral RNA was synthesized in HeLa cells and comparison with the infection of chick embryo fibroblasts showed that similar amounts of virus-specific RNA were made. It seems unlikely, therefore, that the failure to produce infectious virus in HeLa cells is a result of decreased synthesis of viral RNA per host cell. The accumulation of viral RNA eliminates explanations for this nonpermissiveness based on the rapid destruction of newly synthesized viral RNA. The possibility of continuous uncontrolled RNA synthesis was ruled out by the finding that synthesis was completed by 15 hours post infection. Since the number of double-stranded RNA molecules synthesized equals at least the number of single-stranded segments reported in the influenza

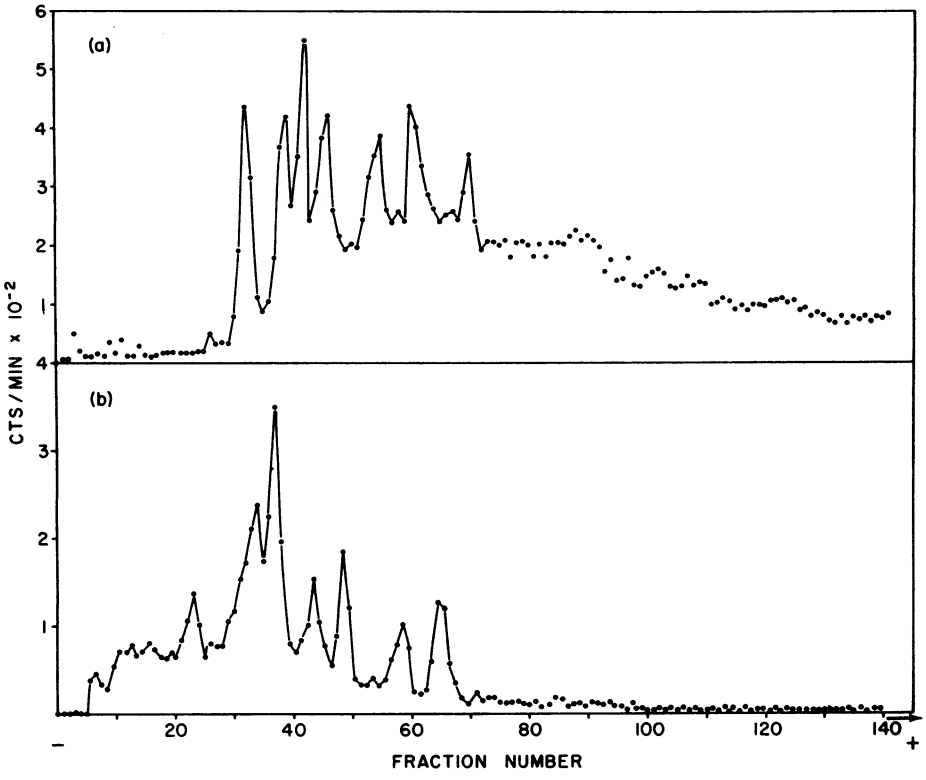

FIG. 6.—Acrylamide gel analysis of double-stranded viral RNA. Cells were infected and labeled with radioactive uridine as described in Fig. 2. Double-stranded viral RNA was prepared by either digestion with RNase (panel *A*) or by 2 *M* NaCl fractionation (panel *B*) (see *Materials and Methods*). The RNA was separated in 15 cm 2.1% agarose-acrylamide gels for 17 hr at 8 ma/gel and the radioactivity in one mm gel slices was determined.

virion,[13, 14] it is reasonable to conclude that the complete viral genome is synthesized. The detection of seven pieces of double-stranded RNA by treatment with RNase or 2 *M* NaCl fractionation instead of four or six[15, 16] may be because of better resolution of RNA in the acrylamide gel system used in this investigation.

There is ample evidence regarding the mode of replication of single-stranded RNA viruses (see review, ref. 17). The input strands are duplicated (RF) and single-stranded pieces are copied from this template (RI) and then released. Our data indicated that this process occurred in infected HeLa cells. The distribution of RNase-resistant RNA in sucrose gradients was similar to that reported for RI, double-stranded pieces of viral RNA were synthesized (RF), and the 20*S* and 14*S* RNA which have been shown to be single-stranded in the permissive infection of chick embryo fibroblast cells accumulated. It is conceivable, however, that the 20*S* and 14*S* RNA represented not only the synthesis of single-stranded progeny (plus strand) but also the accumulation of negative strands. This possibility is being investigated.

The apparently normal synthesis and accumulation of influenza RNA in HeLa

cells suggests that the lack of formation of infectious virus could be the result of incomplete assembly of the virus in this nonpermissive host. Consistent with this interpretation is the reported failure of the nucleocapsid protein of influenza virus to disperse from the nuclear region in this cell. Increased amounts of RNase-resistant RNA were observed during the synthesis of phage F_2 RNA in *E. coli* under conditions which prevented viral assembly by restricting the synthesis of coat protein.[18] Similarly we have found a 5- to 10-fold increase in the percentage of RNase-resistant RNA greater than that reported for the permissive infection of chick embryo fibroblast cells by influenza virus. Thus, the determination of the degree of viral assembly in HeLa cells may offer a more complete understanding of this nonpermissive infection. In addition, this system may prove to be a source for the accumulation of intermediates for a study of the assembly of a multisegmented single-stranded RNA virus.

* This investigation was supported in part by the U.S. Public Health Service research grants RO1-CA 11181 and PO1-CA 10815 from the National Cancer Institute.

† Recipient of U.S. Public Health Service Research Career Development Award 1-K4-CA 42,385 MBY from the National Cancer Institute.

[1] Henle, G., A. Girardi, and W. Henle, *J. Exptl. Med.*, **101**, 25 (1955).

[2] White, D. O., H. M. Day, E. J. Batchelder, I. M. Cheyne, and A. J. Wansbrough, *Virology*, **25**, 289 (1965).

[3] Meulen, V. T., and R. Love, *J. Virol.*, **1**, 626 (1967).

[4] Franklin, R. M., and Breitenfeld, *Virology*, **8**, 293 (1959).

[5] Hillis, W. D., M. A. Moffat, and O. A. Haltermann, *Acta Path. Microbiol. Scand.*, **50**, 419 (1960).

[6] Pristosova, S., J. Lesso, and J. Syanto, *Acta Virol., Prague*, **2**, 177 (1966).

[7] Eagle, H., *Science*, **130**, 432 (1959).

[8] Hecht, L. I., M. L. Stephenson, and E. P. C. Yamercnik, these PROCEEDINGS, **45**, 505 (1959).

[9] Tamoki, T., and G. C. Mueller, *Biochem. Biophys. Res. Commun.*, **9**, 451 (1962).

[10] Pons, M., *Virology*, **33**, 150 (1967).

[11] Martin, R. G., and B. N. Ames, *J. Biol. Chem.*, **236**, 1372 (1961).

[12] Watanabe, Y., S. Millward, and A. F. Graham, *J. Mol. Biol.*, **36**, 107 (1968).

[13] Duesberg, P. H., and W. S. Robinson, *J. Mol. Biol.*, **25**, 383 (1967).

[14] Pons, M. W., and G. K. Hirst, *Virology*, **34**, 386 (1968).

[15] Duesberg, P. H., these PROCEEDINGS, **59**, 930 (1968).

[16] Pons, M. W. and G. K. Hirst, *Virology*, **38**, 68 (1969).

[17] Weissmann, C., *Perspectives in Virology*, (New York: Academic Press, 1967), vol. 5, pp. 1–27.

[18] Lodish, H., and N. Zinder, *Science*, **152**, 372 (1966).