*AN INTERMEDIATE IN THE REPLICATION OF INFLUENZA VIRUS RNA**

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A replicative intermediate (RI) having characteristics of double-stranded RNA with attached nascent single strands has been implicated in the synthesis of single-stranded viral RNA.[1-3] Active participation of such a complex in the formation of single-stranded viral RNA has been demonstrated in cell-free systems with Q-β virus RNA[4, 5] and in infected cells with poliovirus RNA.[6, 7] With more complex viruses, e.g., NDV, such an intermediate complex has not been observed so far. The present report indicates that it occurs during the multiplication of a virus of similar structure, thus indicating its generality in the replication of single-stranded viral RNA.

Materials and Methods.—Virus and cell: The American (Miami) strain of equine influenza virus (EIV) was used. To ensure the absence of defective virus, three virus passages were initiated by inoculating eggs with 1 egg infectious unit (i.u.) each.[12] One hundred i.u. from the third passage were inoculated to make virus stocks which contained 10^9 i.u. and 640 hemagglutinin units (HA) per milliliter.

Primary chick embryo fibroblasts (CEF) were prepared from 10-day-old K-137 chick embryos and cultured in modified Eagle's medium containing 10% calf serum.[8]

General experimental procedure: (a) *Infection at high multiplicity:* For synchronous infection, cells were infected at an input multiplicity of 20–40 i.u./cell at 37° for 50 min. The infected cells were incubated in 5 ml of modified Eagle's medium containing 5% dialyzed calf serum at 37° in a CO_2 incubator.[8] At 3 hr postinfection, actinomycin D was added to a final concentration of 2 μg/ml and 30 min later tritium-labeled uridine (H^3-UR) was added to the desired concentration. At specified time intervals the cells were dispersed with trypsin and suspended in NTE buffer (pH 8.5) [NaCl 0.1 *M*, Tris (pH 8.5) 0.02 *M*, EDTA 0.001 *M*]. RNA was extracted by the phenol-SDS method.[9] After 2 alcohol precipitations the RNA was dissolved in NTE (pH 7.4) and analyzed by velocity sedimentation in sucrose gradients containing the same buffer. RNase resistance was determined in 2 × SSC with 100 μg/ml of RNase for 30 min at 37°. TCA-precipitable radioactivity was determined in scintillation fluid with a toluene base in an Ansitron liquid scintillation spectrometer. The amount of infectious virus produced was determined by inoculating serial tenfold dilutions into 10-day-old chick embryos.[10, 11] Under these conditions only 10^5 i.u. having an HA titer of 320 are produced per culture within 8 hr after infection.

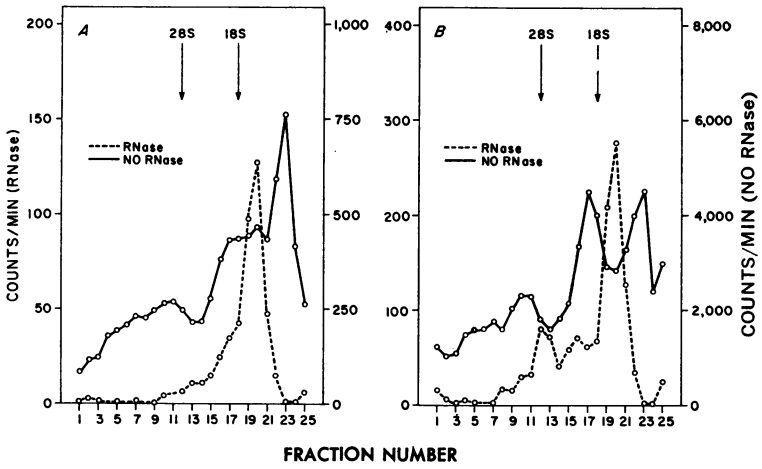
(b) *Infection at low multiplicity:* Cells were infected at input multiplicities of 0.05–0.1 i.u./cell. The infected cells were incubated for 16 hr, at which time they were treated with actinomycin D and labeled with H^3-UR as in (*a*). Each infected culture produced about 10^8 i.u. having an HA titer of 100 within 16 hr after infection.

Materials: Chromatographically purified ribonuclease A (RNase) was purchased from Worthington Biochemical Corp., Freehold, N.J. Tritiated uridine (H^3-labeled at 5 position, spec. act. 20 c/mmole) was a product of Schwarz BioResearch, Inc. SSC contained NaCl 0.15 *M* and Na citrate (pH 7.0) 0.015 *M*. Actinomycin D was a gift from Merck Sharp and Dohme Research Lab.

Results.—Virus-specific RNase-resistant RNA in infected cells: CEF cells infected with EIV at high multiplicity of infection (MOI), and labeled with

H^3-UR in the presence of actinomycin D from three to seven hours after infection, synthesize mostly single-stranded RNA.[12] Approximately 5 per cent of the (labeled) virus-specific RNA synthesized during that time interval is RNase-resistant.[12] The following experiments were carried out to determine whether this RNase-resistant RNA is part of a replicative intermediate similar to that found in cells infected with small RNA viruses.[1-3]

If it is an intermediate in viral RNA synthesis, the RNase-resistant RNA should be preferentially labeled after a short period of exposure to H^3-UR. Accordingly, CEF cells infected at high MOI with EIV were labeled for only ten minutes in the presence of actinomycin D, as described in *Materials and*



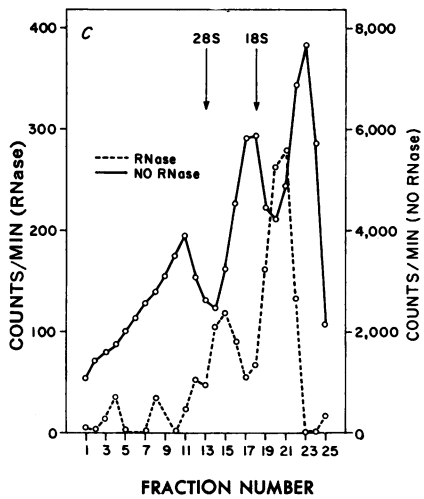


FIG. 1.—Sucrose velocity gradients of virus-specific RNA. Three hr after infection at high MOI, CEF cells were exposed to H^3-UR (250 μc/plate) in 5 ml of medium in presence of actinomycin D (2 μg/ml) for 10 min. Extracted RNA was centrifuged in 5–20% sucrose gradients containing NTE buffer (pH 7.4) at 50,000 rpm for 2 hr in a Spinco SW50 rotor at 4°. Fractions were collected from the bottom of the tube using a Buchner piercing unit. Aliquots of each fraction were tested for OD at 260 mμ, total TCA-precipitable radioactivity (*solid line*), and TCA-precipitable radioactivity after RNase treatment (*broken line*). Background was subtracted from all counts and each fraction was normalized using the OD of 28*S* ribosomal RNA. In addition, 1.8% of the total radioactivity was deducted from the RNase-resistant counts to correct for the enzyme resistant residual cores. (*A*) RNA extracted from cells immediately after 10 min of labeling. (*B*) RNA extracted from cells after 1 hr of incubation in cold medium following 10 min of exposure to H^3-UR. (*C*) RNA extracted from cells after 4 hr of incubation in cold medium following 10 min of exposure to H^3-UR.

Methods. Virus-specific RNA was extracted and analyzed in sucrose gradients by velocity sedimentation. Each fraction was tested for TCA-precipitable radioactivity and for RNase resistance. As shown in Figure 1*A*, an RNase-resistant peak of approximately 14*S* is present. In the peak fraction, 27 per cent of the label is RNase-resistant; in two other experiments, 32 per cent and 38 per cent of the peak fraction was RNase-resistant. If the amount of radioactivity in all the fractions, excluding sRNA, is totaled, 12–15 per cent of the newly synthesized (labeled) RNA is found to be RNase-resistant.

Characterization of 14S RNase-resistant RNA: The 14*S* RNase-resistant RNA could be either RI, or double-stranded RNA, or both. Two other possible explanations of its nature can be eliminated: (1) it does not consist of enzyme-resistant cores that arose by RNase digestion of essentially single-stranded 34*S*, or 18*S*, virus-specific RNA (under our experimental conditions, 1.8% of the single-stranded, normal, cellular RNA and 3% of the RNA isolated from purified virions[12] are found to be RNase-resistant), (2) nor does it consist of double-stranded RNA artificially produced during the experimental manipulations. The reasons are as follows: the peak of this RNA species does not coincide with the peaks of the various single-stranded RNA species (34*S* or 18*S*). After velocity sedimentation the residual RNase-resistant cores display a $s_{20,w}$ of approximately 5*S*. In addition, the proportion of RNase-resistant RNA to total RNA decreases with increased duration of exposure to radioactive label.

14*S* RNase-resistant RNA (Fig. 1*A*) has the characteristics of RI. (1) The profile of RNase resistance in the sucrose gradient is heterogeneous with a broad tail toward the higher *S* values similar to that previously reported for RI[3] but not to that of totally double-stranded RNA.[13] (2) Totally double-stranded RNA is soluble in 2 *M* NaCl, whereas RI is insoluble. Virus-specific RNA was labeled for increasing time intervals, extracted, and treated with 2 *M* NaCl. The precipitate was dissolved and analyzed in a sucrose velocity gradient, and each fraction was analyzed for OD, and RNase resistance. Results obtained after 10 minutes, 20 minutes, and 3 hours of labeling are presented in Figure 2. The profile of RNase-resistant RNA is similar to that of Figure 1*A* and has the characteristics of RI.[3] The supernate was also analyzed for the presence of double-stranded RNA after sucrose velocity sedimentation. After 10 minutes of labeling, 1.5–2.5 per cent of the RNase-resistant RNA was soluble in 2 *M* NaCl. As will be seen later (Fig. 6), the fraction of salt-soluble RNase-resistant RNA increased with increasing duration of labeling. (3) Characteristically, RNase treatment of RI produces RNase-resistant double-stranded RNA the size of which depends on the RNase concentration used.[14] If 14*S* RNA isolated from fractions 19 and 20 (Fig. 1*A*) was treated mildly with RNase (0.2 μg/ml for 15 min at 37°) in SSC and analyzed by velocity sedimentation centrifugation in a sucrose gradient, two peaks of radioactivity were observed (Fig. 3). The faster component has an approximate $s_{20,w}$ of 11*S* and is 85–100 per cent resistant to further RNase treatment as judged by conversion to acid-soluble material. When the 14*S* RNA was treated with a higher concentration of RNase (50 μg/ml at 37° for 15 min in SSC), the $s_{20,w}$ of the RNase-resistant peak was reduced to

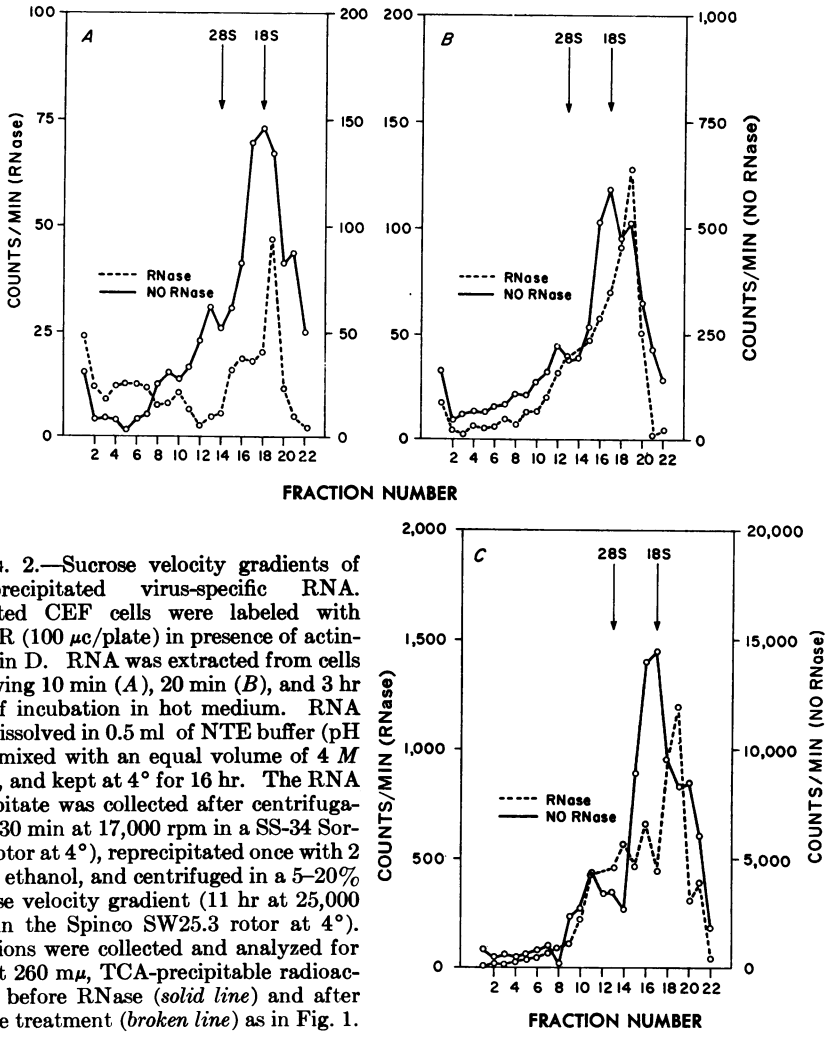


FIG. 2.—Sucrose velocity gradients of salt-precipitated virus-specific RNA. Infected CEF cells were labeled with H^3-UR (100 μc/plate) in presence of actinomycin D. RNA was extracted from cells following 10 min (*A*), 20 min (*B*), and 3 hr (*C*) of incubation in hot medium. RNA was dissolved in 0.5 ml of NTE buffer (pH 7.4), mixed with an equal volume of 4 *M* NaCl, and kept at 4° for 16 hr. The RNA precipitate was collected after centrifugation (30 min at 17,000 rpm in a SS-34 Sorvall rotor at 4°), reprecipitated once with 2 vol of ethanol, and centrifuged in a 5–20% sucrose velocity gradient (11 hr at 25,000 rpm in the Spinco SW25.3 rotor at 4°). Fractions were collected and analyzed for OD at 260 mμ, TCA-precipitable radioactivity before RNase (*solid line*) and after RNase treatment (*broken line*) as in Fig. 1.

7*S* which was also 85–100 per cent resistant to further RNase treatment. The RNase-resistant RNA (11*S* and 7*S*) became completely susceptible to digestion by RNase after heating in 0.1 *M* NaCl at 100° for five minutes followed by quenching in ice water. The sedimentation coefficient of the RNase-resistant RNA (11*S* and 7*S*) in sucrose velocity gradients was not affected by the salt concentration. On the other hand, the sedimentation coefficient of 14*S* RNA depended on the salt concentration, though to a lesser extent than that of single-stranded RNA (34*S* and 18*S*). This suggests the existence in 14*S* RNA of single strands attached to a double-stranded structure which has breaks in only one of the two strands. RNase treatment digests the single strands and causes complete breaks in the double-stranded portion of the complex by digesting the single strand opposite the single breaks. The resulting material (7*S*)

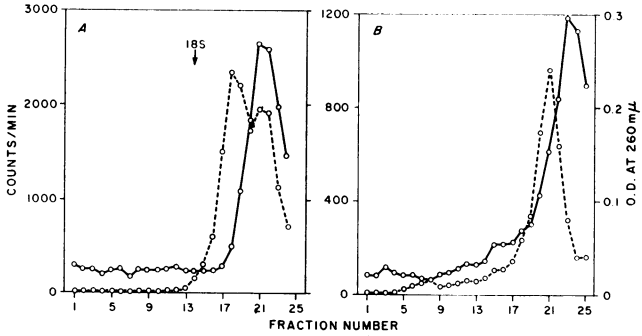


FIG. 3.—Velocity sedimentation profile of H^3-labeled 14*S* RNA after treatment with different concentrations of RNase. RNA (14*S*) isolated from fractions 19 and 20 (Fig. 1*A*), was treated with RNase 0.2 μg/ml (*A*) or 50 μg/ml (*B*) for 15 min at 37°, and analyzed in 5–20% sucrose velocity gradients (4 hr at 36,000 rpm in a Spinco SW39 rotor at 4°). Fractions were collected and analyzed for OD at 260 mμ and TCA-precipitable radioactivity. The arrow shows the position of 18*S* ribosomal RNA determined by OD in a similar gradient run in another bucket of the same rotor.

is completely double-stranded, whereas the 11*S* material still has some single breaks in an essentially double-stranded molecule. For comparison, double-stranded structures consisting of complete plus and minus strands obtained by annealing have a $s_{20,w}$ of 12*S*.[12] (4) The melting curve of the RNase-resistant RNA (7*S*) was determined by measuring the proportion converted to acid-soluble material by RNase after heating at a given temperature for five minutes followed by rapid cooling. Thermal transition profiles at two different salt concentrations are presented in Figure 4*B*. The *Tm* was 81° in 0.1 × SSC and 93° in SSC. The melting curve was slightly broader and the *Tm* was slightly lower than that of a double-stranded RNA molecule of 12*S* consisting of complete plus and minus strands (Fig. 4*A*) produced by annealing of virus-specific RNA isolated from infected cells.[12]

Kinetics of labeling of RI: The kinetics of synthesis of RI were studied in two types of experiments. First, infected cells were labeled continually in the presence of actinomycin D, and RNA was extracted after labeling periods of 10, 20, 60, 120, and 180 minutes. An aliquot of the extracted virus-specific RNA was treated with 2 *M* NaCl in Tris-EDTA buffer (pH 7.4) overnight to separate single-stranded RNA and RI from double-stranded RNA and sRNA. Total RNA, salt-precipitated RNA, and salt-soluble RNA were analyzed in sucrose velocity gradients. Each fraction was tested for OD, and for radioactivity before and after RNase treatment in 2 × SSC. The amounts of total RNA, single-stranded RNA, RI, and totally double-stranded (salt-soluble) RNA (DS) were determined. The results presented in Figure 5 show that there is continuous synthesis of single-stranded virus-specific RNA, of RI, and of DS, even after three hours of labeling. There is a 60-fold increase in the amount of label in total virus-specific RNA as the duration of labeling is increased from ten minutes to three hours. This is to be expected, since even after six hours of

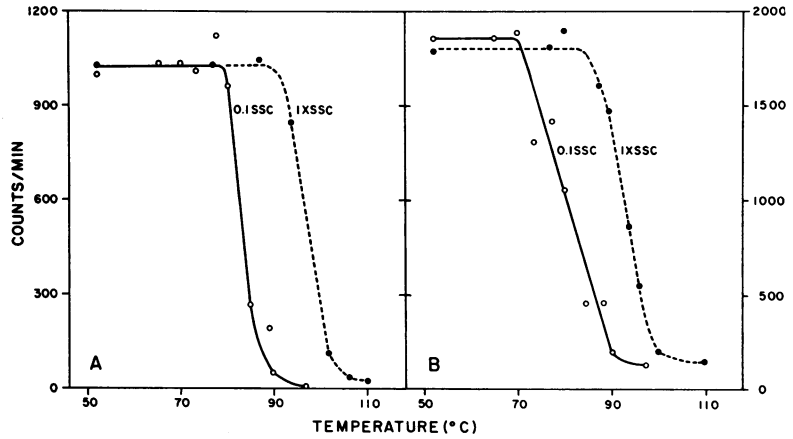


FIG. 4.—Thermal denaturation of (7*S*) double-stranded RNA obtained by RNase treatment. RNase-resistant RNA (7*S*) was obtained by RNase treatment of RI and isolated as in Fig. 3*B*. Aliquots (0.1 ml), in 0.1 SSC or SSC, were heated in sealed ampoules for 5 min at the desired temperature and chilled rapidly in ice water. After treatment with RNase (2 × SSC with 100 μg of RNase in1 ml at 37° for 30 min), TCA-precipitable radioactivity was determined. Double-stranded RNA (12*S*) was obtained by annealing virus-specific RNA and RNAse treatment and isolated after sucrose velocity gradient centrifugation. (*A*) Thermal denaturation of 12*S* double-stranded RNA. (*B*) Thermal denaturation of 7*S* double-stranded RNA.

labeling the intracellular, TCA-soluble, H^3-UR pool has still not reached saturation (unpublished observation). Under conditions similar to those used in the above experiment but without actinomycin D, uninfected CEF cells show a sixfold increase in the amount of H^3-UR in the TCA-soluble pool and a sixteen-fold increase in the amount of label in cellular RNA. Addition of actinomycin D (2 μg/ml) did not affect the entrance of H^3-UR into the intracellular acid-soluble pool, whereas cellular RNA synthesis was inhibited by over 96 per cent (unpublished observation).

The amount of label in RI increases more slowly than the amount of total label. As seen in Figure 6, the fraction of RNA which is RNase-resistant in the 14*S* peak decreased from 37 per cent after ten minutes of labeling to 11 per cent after three hours. On the other hand, the relative proportion of DS to salt-precipitable RNase-resistant RNA increases with duration of labeling; it is 2.5 per cent after ten minutes and 17 per cent after three hours (Fig. 6).

In the second type of experiment, a quasi pulse-chase experiment was carried out. A true pulse-chase experiment is not feasible because H^3-UR enters into, and chases from, the intracellular acid-soluble pool too slowly (unpublished observation). If uninfected CEF cells are labeled with H^3-UR for ten minutes, washed three times, and incubated in presence of cold UR, 50 per cent of the label is still present in the pool one hour later and 30 per cent is still present four hours later. Addition of actinomycin D does not affect the chase of label from the pool. In the absence of actinomycin D the amount of H^3-UR in TCA-precipitable cellular RNA still increases 2.2-fold within four hours after a ten-minute pulse of label, followed by washing and incubation of cells with cold UR.

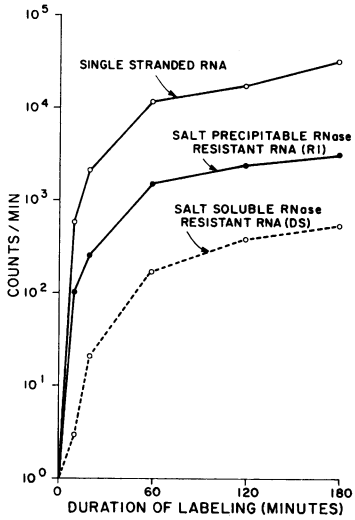


FIG. 5.—Incorporation of radioactivity into various classes of virus-specific RNA. Infected CEF cultures were labeled with H^3-UR from 3.5 hr p.i. in presence of actinomycin D. RNA was isolated from the cells at different time intervals. Salt-soluble and salt-precipitable RNA was isolated after treatment with 2 *M* NaCl as in Fig. 2. The salt-soluble and salt-precipitable RNA fractions were centrifuged in sucrose gradient at 49,000 rpm for 2 hr at 4°. Fractions were analyzed for OD at 260 mμ, and TCA-precipitable radioactivity before and after RNase treatment. The cpm were normalized using OD 260 and corrected for RNase-resistant cores as in Fig. 1. The amount of single-stranded RNA was determined by subtracting RNase-resistant RNA from total RNA.

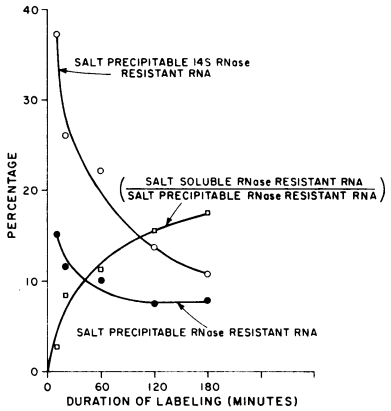


FIG. 6.—Kinetics of incorporation of radioactivity into various classes of virus-specific RNase-resistant RNA. The results were calculated from 3 experiments similar to the one presented in Fig. 5.

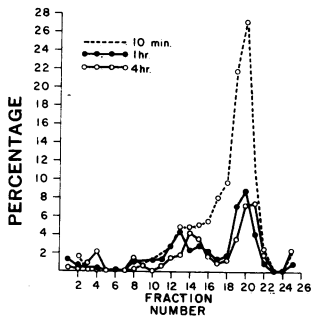


FIG. 7. — Proportion of RNase - resistant RNA in the various fractions of the quasi pulse-chase experiment presented in Fig. 1.

Three infected CEF cultures were incubated in the presence of H^3-UR (250 μc/plate) and actinomycin D for ten minutes. Cells from one plate were harvested immediately and RNA was extracted (10 min). The two remaining cultures were washed three times with 10 ml of phosphate-buffered saline containing unlabeled uridine (2×10^{-4} *M*) and actinomycin D (2.0 μg/ml) and further incubated in cold medium. RNA was extracted after one hour and four hours of incubation. RNA from all three cultures (10 min, 1 hr, and 4 hr) was then analyzed in sucrose velocity gradients and each fraction was tested for total, and RNase-resistant TCA-precipitable radioactivity. The results are presented in Figures 1*A*–*C*. There is a peak of RNase-resistant RNA of 14*S* in all three RNA preparations. The amounts of 18*S*, and of 34*S* single-stranded RNA which are relatively small at the end of a ten-minute labeling period (Fig.

1A) increase tenfold during the next four hours of incubation. On the other hand, the absolute amount of RNase-resistant 14S RNA increases only 3.6-fold during the same four hours of incubation in unlabeled medium. Furthermore, the proportion of 14S RNase-resistant RNA decreases upon further incubation after removal of label as evidenced in Figure 7.

Virus-specific RNA after infection at low MOI: The above observations were made after infection of CEF cells at high MOI which causes production of noninfectious viral particles. To determine whether the same events took place in the normal replication process, when the cells produce infectious virus, similar experiments were repeated using primary CEF cells which had been infected at a MOI of 0.05–0.1 i.u./cell. The infected cells were labeled with H^3-UR at 16 hours after infection either for 10 minutes, or for 4 hours, or for 10 minutes followed by 4 hours in cold medium. The profile of intracellular virus-specific RNA was the same as that obtained at high MOI. Both 34S and 18S single-stranded RNA's and 14S RNase-resistant RNA were found. Toward the end of the infectious cycle (24 hr after infection) there was also an increase in amount of double-stranded RNA with the characteristics of complete double-stranded 12S molecules. Apparently this RNA species does not take part in the formation of viral RNA.[15]

Conclusions.—Most (90%) of the virus-specific RNA (34S and 18S) synthesized between 3.5 and 7.5 hours after infection in the presence of actinomycin D consists of plus strands; the rest are minus strands.[12] The 34S RNA appears to be caused by aggregation of 18S RNA molecules.[12] It has been shown in the present study that infected cells also synthesize a 14S partially RNase-resistant RNA which behaves like a precursor of the 18S (and 34S) virus-specific RNA. Formation of a double-stranded RNA species after RNase treatment of RI, dependence of size of the double-stranded RNA on RNase concentration, and precipitation in 2 *M* NaCl indicate that most of the 14S RNA complexes are RI's. A small fraction of duplex molecules are also present in infected cells; their concentration increases with the duration of labeling. The RNA species present in cells infected at high MOI (noninfectious virus produced) and in cells infected at low MOI (infectious virus produced) are similar.

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