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Synthesis of Saint Louis Encephalitis Virus Ribonucleic Acid in BHK-21/13 Cells

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Infection of baby hamster kidney cells (BHK-21/13) with Saint Louis encephalitis (SLE) virus depressed the rate of protein and ribonucleic acid (RNA) synthesis until viral RNA synthesis began 6 hr postinfection (PI). Virus-directed RNA synthesis was subsequently inhibited until 12 hr PI when virion maturation began. The rate of protein synthesis reached a peak 6 hr PI and was subsequently depressed until just before the onset of virion maturation. Density gradient analysis of phenol-extracted RNA from actinomycin-treated infected cells indicated that, at 6 to 8 hr and again at 12 to 20 hr PI, three species of viral-specific RNA were synthesized. The most rapid sedimenting form (43*S*) was ribonuclease-sensitive and had a base composition similar to the RNA isolated from mature virions. The 20*S* RNA species was ribonuclease-resistant and had a sedimentation coefficient and base composition similar to the replicative form associated with other arbovirus infections. The 26*S* RNA was ribonuclease-resistant (0.2 μg/ml, 0.1 M NaCl, 25 C, 30 min) and had a nucleotide base composition closer to the 20*S* form than to the values for 43*S* RNA. Five-minute pulse labeling of infected cultures during the period viral RNA synthesis was maximal resulted in labeling of only the 20S to 22S RNA fractions. With pulse-labeling periods of 10 min, both the 20*S* and 26*S* RNA species were radioactive. Periods of radioactive labeling of as long as 15 min were required before the 43*S* form was radioactively labeled. These results suggest that the 20*S* and 26*S* RNA may be intermediate forms in the synthesis of 43*S* viral RNA.

The synthesis of viral-directed ribonucleic acid (RNA) in cells infected by group A arboviruses has been studied intensively (8–10, 17, 20, 21, 28, 29). Replication of viral RNA by these viruses is initiated within the 2nd hr after infection, with the formation of three distinct types of RNA which have sedimentation coefficients of approximately 40*S*, 26*S*, and 20*S*, respectively (10, 17, 20, 27–29). It is generally agreed that the 40*S* RNA is infectious and represents newly synthesized single-stranded RNA to be incorporated into progeny virions (10, 20, 27, 28). The nature and function of the 20*S* and 26*S* RNA associated with the synthesis of group A arbovirus RNA are not yet thoroughly understood (8, 9, 27, 28).

In contrast to the rapid replication of the group A arboviruses, the formation of complete dengue (31) and Saint Louis encephalitis (SLE) virus, a group B arbovirus, does not begin until 12 hr after infection. This feature of SLE virus replication raises a number of questions about its mechanism of replication, since its general properties and genome are similar to those reported for group A arboviruses (10, 22, 28). This report represents the beginning of a study on the biochemistry of SLE virus replication in BHK-21/13 cells. In this communication, we report some of the general features of SLE viral RNA synthesis in the infected cells.

MATERIALS AND METHODS

Cells and media. Baby hamster kidney cells (BHK-21/13; 15) obtained from the American Type Culture Collection were grown in a lactalbumin tryptose-phosphate broth medium (23) modified by the addition of yeastolate to a final concentration of 0.1%.

The stable pig kidney cell line designated PS (13), used for virus production and plaque assay, was received from E. G. Westaway. These cells were grown in monolayer roller bottle cultures in medium 199 (18) supplemented with 10% calf serum.

All cell growth medium contained 200 units of penicillin per ml, 100 μg of streptomycin per ml, and 5 μg of fungizone per ml.

Virus. The large plaque mutant of the P-15 strain of SLE virus (19) was obtained from W. McD. Hammon of the University of Pittsburgh. Before use in these experiments, the virus was passed eight times and plaque purified twice in PS cells. Seed virus was prepared by inoculating PS cultures in cylindrical roller

bottles at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU) per cell. Adsorption was allowed to take place at 37 C for 1.5 hr, fluid containing the unadsorbed virus was removed, and growth medium containing 2.1 mg of $NaHCO_3$ per ml was added. Thirty-six hours later, cells and supernatant fluid were harvested and the cells were disrupted at 20 kc with a Biosonik II sonic oscillator for 1 min at 4 C. The virus suspension was clarified by centrifugation at 8,000 × *g* for 15 min and stored at −70 C.

Virus assay. The infectivity of SLE virus preparations was assayed by plaque formation on monolayers of PS cells (33).

Hemagglutination titers (HA) of virus were determined by use of gander erythrocytes as described by Clarke and Casals (5).

Determination of infective centers. For infective center assay, confluent BHK-21 cell monolayers were infected with SLE virus at MOI values ranging from 5.2 to 97.1. Virus was allowed to adsorb for 1.5 hr at 37 C, the monolayers were rinsed three times with phosphate-buffered saline (PBS), growth medium was added, and the cultures were incubated at 37 C for 4 hr. The medium was then removed, the cultures were washed three times with PBS and trypsinized, and the cells were enumerated with the aid of a hemocytometer. The cells in suspension were washed two times with PBS by centrifugation and were resuspended in 5 ml of SLE antiserum diluted 1:10 in medium 199 without serum. This antiserum had a titer of 1:320 by neutralization test against 100 PFU of virus. The cell-antiserum mixture was incubated at room temperature for 30 min, cells were diluted in growth medium, and 0.2-ml volumes were plated on monolayers of PS cells. A 0.5-ml amount of overlay medium was added to fix the cells to the PS cell monolayer, and the cultures were overlaid for plaque formation.

Assay of infectious RNA. Monolayer cultures of PS cells were washed with warm PBS and then were osmotically shocked by successively washing with PBS which contained 0.3, 0.6, and 0.9 M Na_2SO_4. RNA was diluted in ice-cold Ca^+- and Mg^+-ion-deficient PBS which contained 500 μg of diethyaminoethyl (DEAE)-dextran per ml, and six cultures were inoculated with 0.2 ml of each dilution. Infected cultures were incubated at 27 C for 20 min and then were washed consecutively with PBS which contained 0.6 and 0.3 M Na_2SO_4 and PBS alone. After the last wash, the monolayers were overlaid for plaque formation. The standard deviation of infectious RNA titers obtained by this assay technique was 2.91.

Concentration and partial purification of SLE virus. Monolayers of PS cells were infected at an MOI of 10 in medium containing 0.5 μg of actinomycin D per ml. After adsorption, the cultures were fed with medium which contained 0.5 μg of actinomycin and 0.1 μc of ^{3}H-uridine or 100 μc of ^{32}P per ml and were incubated for 40 hr. Medium and cells were collected, sonic treated, and clarified by centrifugation. Saturated ice-cold ammonium sulfate in 0.1 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (*p*H 7.5) was slowly added to the clarified virus suspension to a final concentration of 2.0 M. The resulting precipitate was centrifuged at 8,000 × *g* for 15 min, dissolved in 10% of the original volume of TE buffer [0.01 M Tris-hydrochloride, 0.001 M ethylenediaminetetraacetic acid (EDTA)], and filtered through a G-25 Sephadex column at 4 C with TE buffer. The virus-rich eluate was added to a magnesium pyrophosphate gel slurry (24). Virus was eluted from the gel with 0.4 M potassium phosphate buffer (*p*H 7.0). A 5-ml amount of this concentrated, partially purified virus was then layered on 35 ml of a linear potassium tartrate gradient (30 to 50%, w/v, potassium tartrate in TE buffer which contained 0.2% bovine albumin) and was centrifuged in the SB-110 rotor of an International B-60 centrifuge at 80,000 × *g* for 20 hr at 4 C. Fractions (1 ml) were collected from the bottom of the tube and were assayed for HA, plaque formation, and radioactivity.

Replication of SLE virus and viral RNA. Monolayer cultures of BHK-21 cells were infected at an MOI of 25 PFU in 0.5 ml and were incubated for 1.5 hr at 37 C for adsorption. The cells were then washed three times with PBS to remove extracellular virus, the growth medium was replaced, and the cultures were incubated at 37 C. At the indicated times, cells from four cultures were scraped off the glass into the medium, and a sample of the suspension was stored at −70 C for assay of viral infectivity. Infectious RNA was extracted from the remainder of the culture with phenol-SDS (17) and was stored at −70 C until assayed for infectivity.

Estimation of rates of protein, RNA, and deoxyribonucleic acid (DNA) synthesis. Normal and infected cultures were pulse-labeled by addition of ^{3}H-uridine, ^{3}H-leucine, or ^{3}H-thymidine (10 μc per culture) for 1 hr at various times after infection. RNA, protein, and DNA were extracted by a modified Schmidt-Thannhauser procedure (25). RNA was estimated by the orcinol method (12), protein content was determined by the method of Lowry et al. (14), and DNA was determined by the diphenylamine reaction (4).

Extraction of RNA from infected cultures and purified virus. BHK-21 cells were infected at an MOI of 20 in the presence of 0.5 μg of actinomycin per ml. At 2- to 4-hr intervals postinfection (PI), cultures were pulse-labeled with either ^{3}H-uridine (10 μc per bottle) for 1 hr or ^{32}P (100 μc per bottle) for 30 min. Cytoplasmic RNA was then extracted from the cultures with SDS-phenol at 37 C by the method of Mecs et al. (17). Phenol was removed from the aqueous phase with four ether extractions, and the RNA was precipitated by the addition of 6 volumes of ice-cold ethyl alcohol. This mixture was maintained at 4 C for 18 hr; the precipitate was dissolved in LTM buffer (11) which contained 1 mg of bentonite per ml and was stored at −70 C until analyzed.

RNA was extracted from purified virus for sucrose density gradient centrifugation analysis with water-saturated phenol at 25 C (32).

Sucrose density gradient sedimentation. A 0.5-mg amount of radioactive RNA dissolved in 0.5 ml of LTM buffer was mixed with 0.25 μg of BHK-21 cell ribosomal RNA and layered over a 12-ml linear sucrose gradient (15 to 30%, w/v, sucrose; 0.1 M NaCl; 0.01 M Tris-hydrochloride, *p*H 7.5; 0.001 M EDTA).

Centrifugation was at 38,000 rev/min (160,000 × *g*) for 20 hr in an SB-283 rotor of an International model B-60 ultracentrifuge.

Fractions of 0.25 ml were collected by piercing the bottom of the tube. RNA in alternate fractions was examined for ribonuclease sensitivity by incubation with 0.2 μg of ribonuclease per ml for 30 min at 25 C. After incubation, 5 mg of bovine albumin was added and the acid-insoluble material was precipitated by addition of an equal volume of 10% trichloroacetic acid. The precipitates were collected on B-6 membrane filters (Schleicher and Schuell Co., Keene, N.H.), washed with 10 ml of 5% trichloroacetic acid, dried, placed in scintillation vials, and covered with 10 ml of toluene scintillation fluid [6.0 g of 2,5-diphenyloxazole, 0.2 g of 1, 4-bis 2-(5-phenyloxazalyl)-benzene per liter of toluene].

Fractions to be examined for optical density (OD) and total radioactivity were treated as follows. A 0.1-ml amount of each fraction was removed and added directly to scintillation vials which contained 10 ml of Triton-toluene scintillation fluid [333 ml of Triton-x-100 (Rohm & Hass Co.); 8 g of 2-(4′-*t*-butyl-phenyl)-5-(4″-biphenyl)-1,3,4-oxidiazole; 0.5 g of 2-(4′-biphenyl)-6-phenyl-benzole; and 667 ml of toluene]. The remaining 0.150 ml was diluted with gradient buffer and analyzed for OD at 260 nm.

Sedimentation values of RNA isolated from infected cells and purified virus were estimated by the method of Martin and Ames (16) with BHK-21 ribosomal RNA as a marker.

Base composition of various types of viral RNA. Various types of viral-specific ^{32}P-labeled RNA from infected cells and partially purified virus were isolated and purified by the methods described above. Base composition analysis of the ^{32}P-labeled RNA was performed by high-voltage paper electrophoresis of alkaline hydrolysates in pyridine-acetate buffer at *p*H 3.5 (26).

Chemicals. Uridine-5-^{3}H (20.1 c/mmole), ^{3}H-leucine (2.0 c/mmole), and ^{3}H-thymidine (5 c/mmole) were purchased from Schwarz Bio Research Inc. Carrier-free ^{32}P was purchased from the Cambridge Nuclear Corp. Bovine pancreatic ribonuclease A was obtained from the Worthington Biochemical Corp. Actinomycin D was obtained through the courtesy of Merck Sharp and Dohme Research Laboratories.

RESULTS

Sensitivity of BHK-21 cells to SLE virus. It was essential to determine the input multiplicity necessary to initiate virus replication under one-step growth curve conditions. To determine the percentage of cells capable of releasing SLE virus, confluent monolayers of BHK-21 cells were infected at input multiplicities ranging from 5.2 to 97.1 PFU per cell. The fraction of cells which formed plaques was not appreciably affected by increasing the input multiplicity (Table 1). Eighty-seven to 95% of the cells from cultures infected at input multiplicities from 5.2 to 97.1 initiated infectious center formation.

Time course of formation of infectious RNA and mature SLE virus. The growth of SLE virus in BHK-21 cells is shown in Fig. 1. The latent period lasted for approximately 11 hr and was followed by a phase of rapid increase in virus from the 12th to 24th hr, at which time the virus titer was 2 × 10^8 PFU per ml. Synthesis of infectious RNA was initiated 6 hr PI and was subsequently delayed until virion maturation began. Of special interest is the observation that synthesis of infectious RNA is initiated 6 hr before the onset of virion maturation and is complete 12 hr before maximum virus titers are produced.

Influence of SLE virus infection on overall synthesis of RNA, DNA, and protein. The effects of SLE virus infection at high multiplicities on synthesis of RNA, DNA, and protein are shown

TABLE 1. *Infectious center formation by BHK-21 cells infected with SLE virus at various input multiplicities*

Input multiplicity (PFU/cell)	Per cent of virus-exposed cells forming infectious centers[a]
5.2	87 ± 9.1
10.4	89 ± 8.0
27.0	92 ± 8.1
46.0	93 ± 7.0
97.1	95 ± 4.0

[a] Values are means and standard deviations of four determinations.

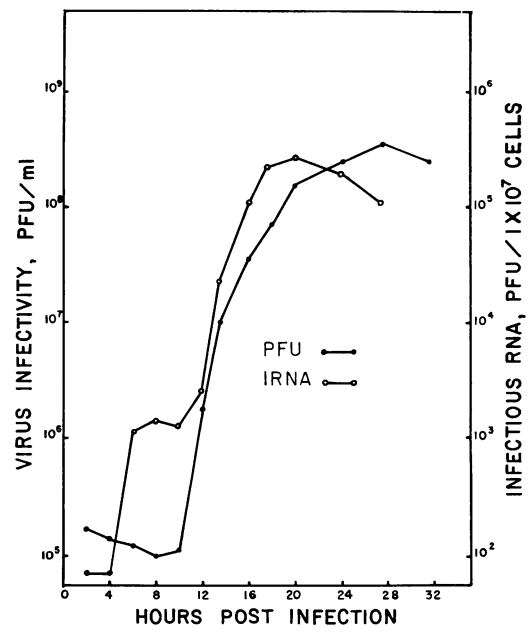

FIG. 1. *Time course of SLE virus and infectious viral RNA synthesis. IRNA, infectious RNA.*

in Fig. 2. The results of these pulse-labeling experiments indicate that, 2 hr after infection at an MOI of 100, protein synthesis is 70% normal and RNA synthesis 60% normal. Infection of cultures at a multiplicity of 50 depressed the rates of protein and RNA synthesis to a greater extent than did infection with 100 PFU per cell. The rate of RNA synthesis in the infected cultures increased sharply at 6 hr PI to a rate 12 times that of the noninfected control. RNA synthesis was then depressed until the 16th hr PI, when it increased to reach a rate nine times the control at 18 hr. The rate of protein synthesis increased gradually from the 2nd hr to reach a rate 1.5 times the control at 8 hr PI. Protein synthesis was depressed until just before the initiation of virion maturation at the 10th hr PI. Maximal rates of protein synthesis were reached 14 hr after infection. The rate of DNA synthesis in the infected cultures did not change significantly until cytopathic changes began to appear 18 hr PI.

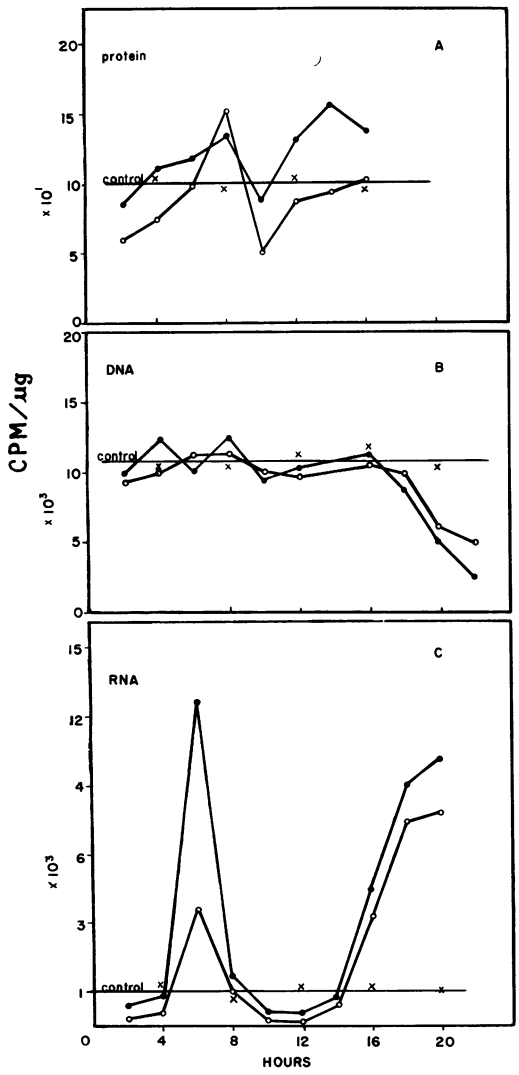

FIG. 2. *Effect of SLE virus infection on the synthesis of protein, DNA, and RNA in BHK-21 cells. Symbols: ○, MOI of 50; ●, multiplicity of infection 100; noninfected control.*

SLE virus growth and RNA synthesis in actinomycin-treated cells. Growth-curve experiments were performed in BHK-21 cultures in the presence of actinomycin D, at concentrations from 0.05 to 1.0 µg per ml, in order to determine whether SLE viral synthesis was suppressed by this inhibitor of DNA-dependent RNA synthesis. The duration of the latent period and the final yield of virus were not affected by actinomycin D at concentrations as high as 0.50 µg/ml (Fig. 3). Therefore, synthesis of viral RNA could be studied under conditions in which cellular RNA synthesis was inhibited.

Figure 3 compares the synthesis of RNA in virus-infected and in noninfected actinomycin-treated cultures. At 8 hr PI, the rate of RNA synthesis in the infected actinomycin-treated cul-

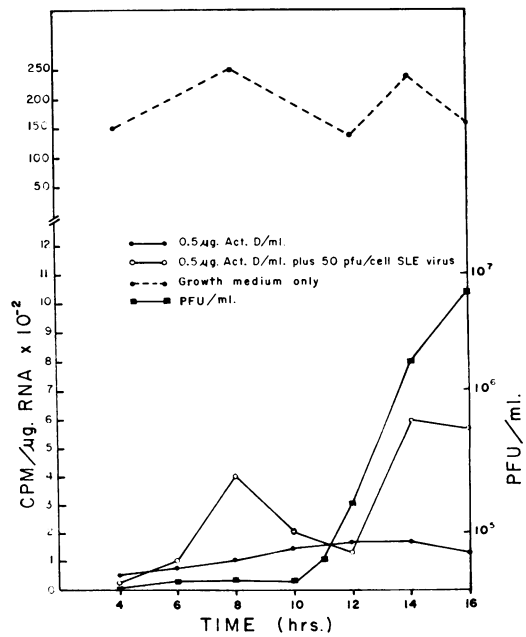

FIG. 3. *Stimulation of RNA synthesis in actinomycin-treated BHK-21 cells after SLE virus infection. Infected cultures containing 0.5 µg of actinomycin per ml were exposed to 10 µc of ³H-uridine for 1 hr at the indicated interval. The RNA was extracted (25) and measured by the orcinol method (12).*

tures was fourfold greater than in the uninfected actinomycin-inhibited culture and one-sixtieth that of nontreated cells. Immediately after the early stimulation, synthesis of RNA in the infected cells was depressed until 14 hr PI, when it had increased to a rate threefold greater than that observed in the cultures treated only with actinomycin.

Comparison of the rates of total RNA synthesis in SLE virus-infected actinomycin-treated (Fig. 3) and actinomycin-free (Fig. 2) cultures reveals that SLE viral RNA synthesis is biphasic, with an early phase beginning during the second half of the latent period and a late phase which is concomitant with virion maturation. Data from repeated experiments indicate that early viral RNA synthesis is delayed for 1 to 2 hr in the actinomycin-treated cultures. However, the late phase of RNA synthesis in the treated and non-treated cultures is initiated at the same time in the infection sequence.

Sedimentation characteristics of SLE viral RNA. A sucrose gradient profile of H^3-uridine-labeled RNA extracted from partially purified SLE virus is shown in Fig. 4, with accompanying 28*S* and 16*S* BHK-21 ribosomal RNA. This gradient contained a total of 7,100 counts/min, of which 5,700 counts/min, approximately 80%, sedimented in a symmetrical peak. Assuming that the sedimentation coefficient is proportional to the distance traveled from the meniscus during centrifugation (16), SLE viral RNA was calculated to have a sedimentation coefficient of 43*S*.

Sucrose gradient analysis of RNA extracted from actinomycin-treated infected cells. Figures 5 and 6 show the results of experiments which characterize the sedimentation coefficients of RNA synthesized during SLE viral replication. The radioactivity profile of RNA from cultures infected for 6 to 12 hr showed three major peaks not observed in the profile of RNA from noninfected cells. The fastest sedimenting peak (43*S*) was ribonuclease-sensitive and identical in its sedimentation characteristics to the RNA isolated from purified virus; presumably, it is newly synthesized progeny viral RNA. The slower sedimenting RNA was resolved into two ribonuclease-resistant species which have sedimentation values of 26*S* and 20*S*, respectively. The 20*S* RNA is presumably a double-stranded molecule similar to the replicative form associated with the synthesis of picornavirus (1–3, 5) and arbovirus (9, 10, 21, 27) RNA. Resistance of the SLE virus-directed 26*S* RNA species, labeled by 1 hr of exposure to ^{3}H-uridine, to hydrolysis by 0.2 μg of ribonuclease per ml indicates that this molecular species is distinct from the 26*S* form

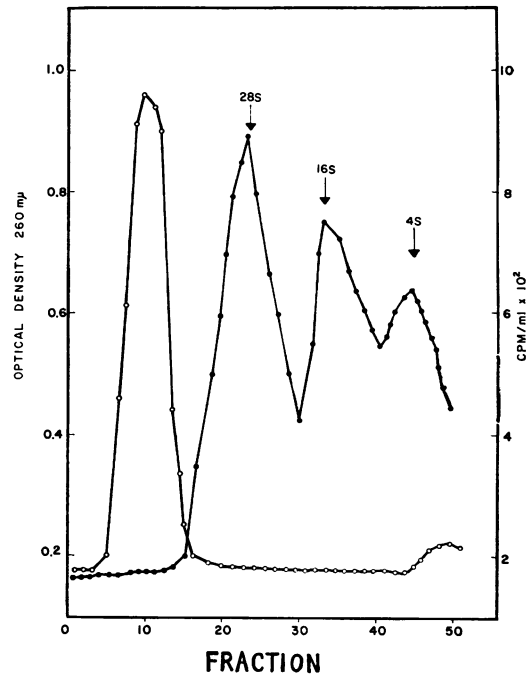

FIG. 4. *Sedimentation profile of ^{3}H-uridine-labeled RNA from purified SLE virus. After sedimentation and fractionation on sucrose gradients, the acid-precipitable radioactivity and OD at 260 nm of each fraction were measured. The OD_{260} markers at 28S, 16S, and 4S are from BHK-21 cells. Symbols: ○, trichloroacetic acid-precipitable counts per ml; ●, OD_{260}. The bottom of the gradient is to the left of this and other figures.*

found in group A arbovirus infections (10, 27–29). Preliminary experiments indicate that the 26*S* RNA labeled early in SLE infection is digested by higher concentrations of ribonuclease (20 μg/ml) to 20*S* and 8*S* fragments (*unpublished data*).

The times of appearance of the 43*S*, 26*S*, and 20*S* forms were analyzed (Fig. 5 and 6). At 6 to 8 hr PI, the 43*S* single-stranded RNA was the most prominant species, although 26*S* RNA and 20*S* RNA were present. From the 10th to 12th hr PI, very small amounts of ^{3}H were incorporated into the 43*S* and 26*S* species, indicating that single-stranded RNA was not being formed, although a large amount of 18*S* to 20*S* RNA was still being synthesized. Beginning at 12 to 14 hr PI, synthesis of 43*S* and 20*S* to 26*S* RNA was again initiated and continued uninterrupted with the formation of large amounts of 43*S* RNA and smaller amounts of the 20*S* and 26*S* ribonuclease-resistant forms. These results are in agreement with the data of Fig. 1 to 3, which indicate that viral RNA synthesis begins 6 hr PI and is subsequently delayed for 4 hr. Formation of large amounts of single-stranded progeny viral RNA

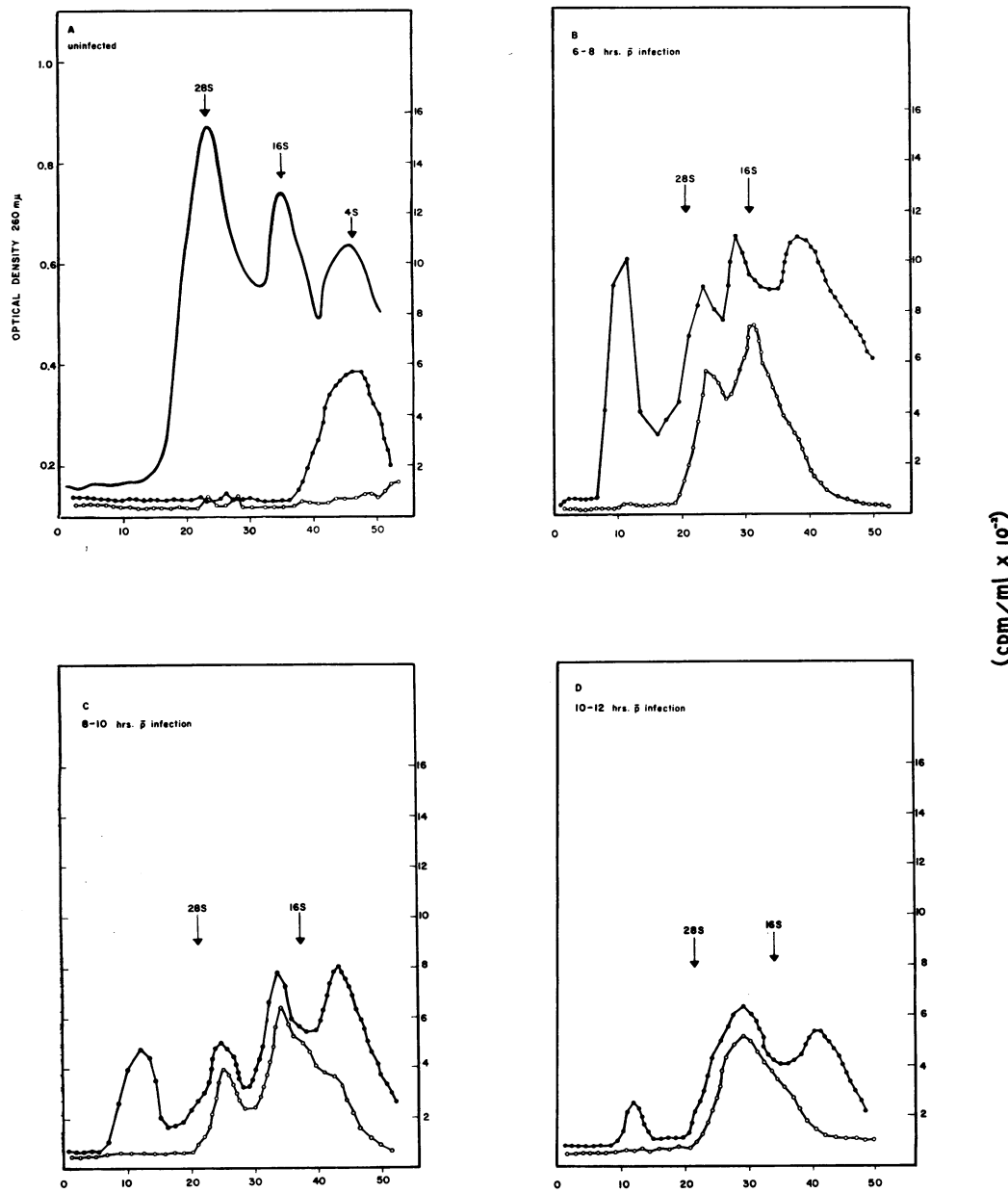

FIG. 5. *Sedimentation profile of* ^{3}*H-uridine-labeled RNA from BHK-21 cells infected with SLE virus in the presence of 0.5 μg of actinomycin D per ml. At the indicated intervals PI, 10 μc of* ^{3}*H-uridine was added for 2 hr; the RNA was extracted by the phenol-SDS method and was sedimented in a 5 to 30% sucrose gradient for 20 hr at 160,000 × g. Fractions were collected and the acid-precipitable radioactivity before (●) and after ribonuclease treatment (○) was determined. The designations 16S and 28S indicate the OD peaks of ribosomal RNA.*

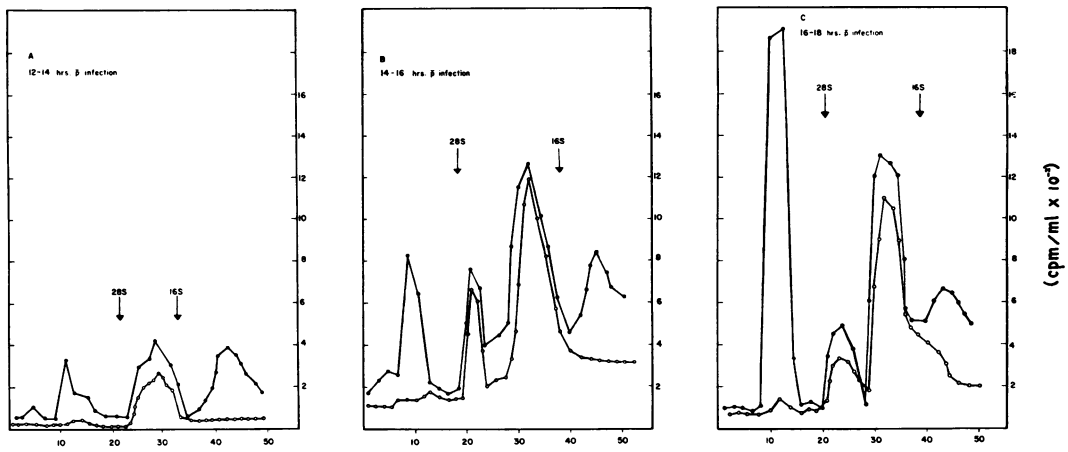

FIG. 6. *Sedimentation profile of ^{3}H-uridine-labeled RNA from SLE virus-infected cells. RNA was labeled, extracted, and sedimented as described in the legend to Fig. 5.* (●) *Trichloroacetic acid-precipitable counts before* (●) *and after* (○) *ribonuclease treatment. The designations 16S and 28S indicate the OD peaks of ribosomal RNA.*

did not begin until viral maturation commenced, about 11 hr after infection.

Sequence of the development of viral RNA forms. Between the 14th and 16th hr PI, when the rate of viral RNA synthesis was maximal, sequential development of the RNA forms was studied by varying the duration of the ^{3}H-uridine pulse (150 μc per bottle) from 2.5 to 15 min (Fig. 7). The RNA from cultures pulsed for 15 min had a radioactive profile similar to that of the cultures pulsed for 1 hr, except that the 26*S* peak was more prominent with the shorter pulse time. The 43*S* RNA peak was less prominent with a 10-min pulse and was not labeled at all in cultures pulsed for 2.5 and 5.0 min, respectively. The only types of RNA taking up the label in cells pulsed for 2.5 min were the ribonuclease-resistant 20*S* to 22*S* forms. Radioactive RNA isolated from the cultures pulsed for 5 min contained both the 20*S* and 26*S* ribonuclease-resistant forms.

Base composition of various types of viral RNA. Viral RNA extracted from ^{32}P-labeled partially purified virus was analyzed for its base content. SLE viral RNA was rich in purine bases, with adenine as the major nucleotide residue (Table 2). The base composition of 43*S* RNA isolated from infected cells during the latent period and that of 43*S* RNA isolated during viral maturation were similar and resemble virion RNA.

The base ratios of 20*S* and 26*S* ribonuclease-resistant RNA were distinct from those observed for 43*S* and virion RNA. The base values obtained for the 20*S* molecules formed early and late were similar and approximate the composition expected for a base-paired molecule (Table 2). The base content of the 26*S* viral-specific ribonuclease-resistant form was different from that observed for 43*S* RNA, 20*S* RNA, or the values calculated for a simple base-paired molecule. For all four nucleotides, the 26*S* RNA was closer to the observed 20*S* base composition values and the values calculated for a duplex or triplet form than to those observed for the 43*S* forms.

DISCUSSION

SLE viral-directed RNA synthesis is initiated 6 hr after infection and is subsequently suppressed until virion maturation begins. It has previously been reported that, during the 12-hr latent phase of type 2 dengue virus, RNA synthesis is required 6 hr before the onset of virion maturation (30, 31). The overall rate of RNA synthesis in the dengue-infected cells is not stimulated until virion maturation begins, thus indicating that either the rate of synthesis or the amount of early RNA required for dengue virus replication is less than that required during the early period before formation of complete SLE virions. The requirement of group B arboviruses for RNA synthesis for an extended period before virion maturation is distinct from the synthesis of group A arbovirus RNA, which is initiated shortly before complete virions are formed and continues uninterrupted during viral replication (20, 27). There are several reasons why group B arboviruses may require RNA synthesis for an extended period before viral maturation. Early formed RNA may (i) be pre-

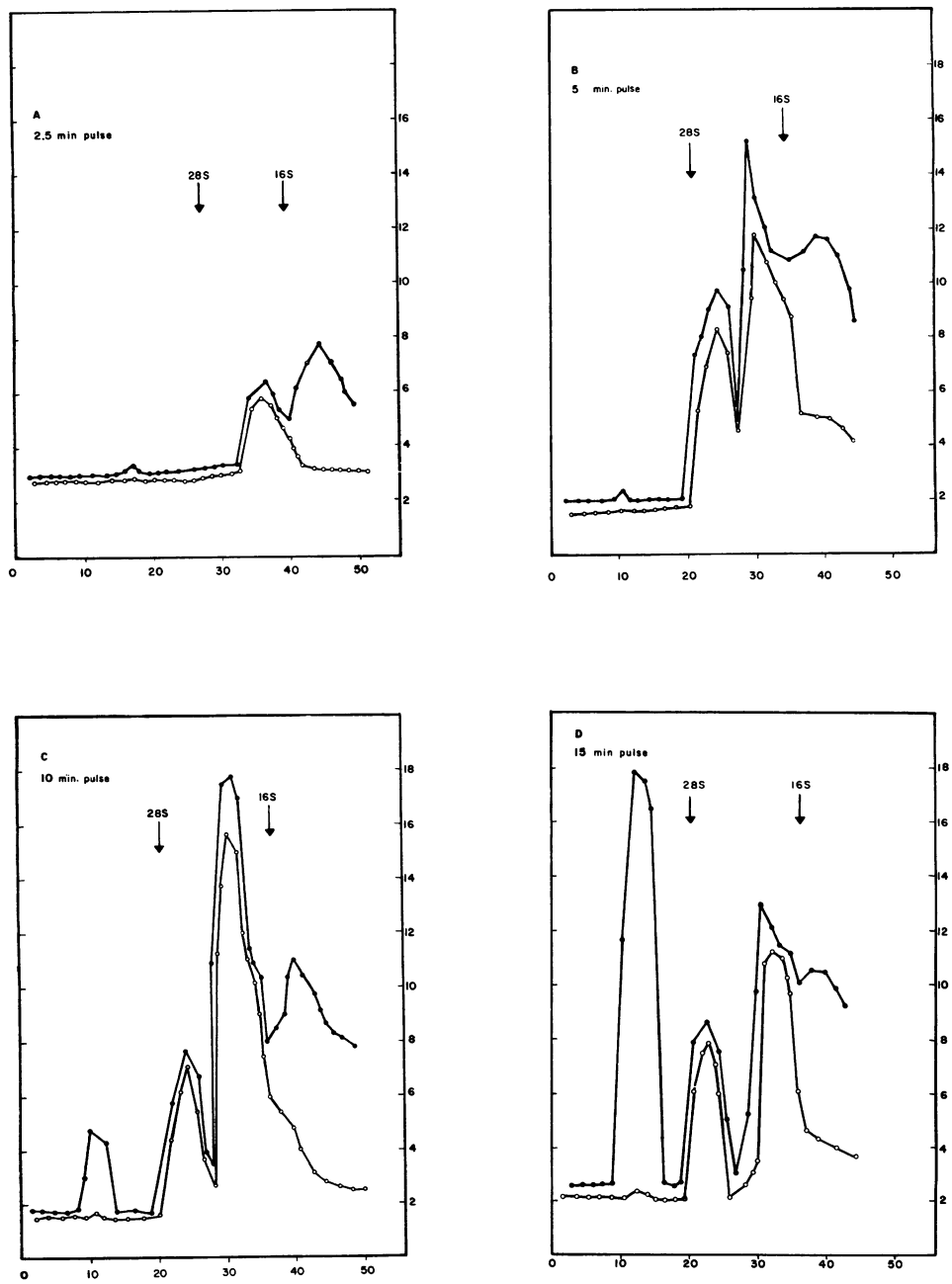

FIG. 7. *Sedimentation profile of ^{3}H-labeled RNA from SLE-virus-infected BHK-21 cells. Actinomycin-treated cells were infected for 16 hr and then were pulse labeled for various intervals; the RNA was extracted. After sedimentation and fractionation on sucrose gradients, the acid-precipitable radioactivity before (●) and after (○) ribonuclease treatment was determined.*

TABLE 2. *Base composition of various types of virus-specific RNA*

Source of RNA	Fraction	Nucleotides per 100 nucleotides[a]			
		Uridine	Guanosine	Cytosine	Adenosine
Purified SLE virus	43S	21.4(±0.2)	26.2(±0.1)	21.7(±0.2)	30.7(±0.4)
Infected cells					
7–10 Hr PI	43S	21.7(±0.5)	26.5(±0.7)	21.5(±0.3)	30.3(±0.4)
	20S	26.4(±0.3)	22.8(±0.5)	24.6(±0.7)	26.2(±0.5)
16–19 Hr PI	43S	21.9(±0.5)	26.8(±0.5)	21.7(±0.4)	29.6(±0.5)
	26S	24.5(±0.3)	23.5(±0.2)	23.6(±0.4)	28.4(±0.5)
	20S	26.8(±0.2)	23.1(±0.3)	23.5(±0.2)	26.6(±0.4)
Calculated values based on viral RNA					
Duplex (1+, −1)	20S	26.1	23.9	23.9	26.1
Triplet (2+, −1)	26S	24.5	24.3	23.1	27.6

[a] Values are means and standard deviations of seven determinations.

dominantly composed of negative strands used for formation of the double-stranded replicative form (RF, 20S species) which is preferentially formed before large amounts of the positive strands are synthesized, (ii) be messenger RNA used to form polyribosomes for the synthesis of viral enzymes and coat proteins required before virion maturation, or (iii) be precursor viral RNA which eventually will be coated. During the early phase of SLE virus RNA synthesis, 43S RNA, 26S RNA, and 20S RNA are formed, although only small amounts of infectious RNA are synthesized. These observations are consistent with the interpretation that early formed 43S RNA is composed of small amounts of positive-strand RNA which is infectious and negative strands utilized for formation of the double-stranded 20S replicative form. The results presented here are not sufficiently precise to differentiate an asymmetric synthesis of the RF form as suggested for dengue virus (30). Preliminary studies with cytoplasmic fractions from SLE virus-infected cells indicate that viral polysomes are formed and are active in protein synthesis 6 hr after infection.

Analysis of the RNA extracted from infected cells revealed that the three species of viral-specific RNA formed during the early and late phases of viral RNA synthesis are similar. The largest species has a sedimentation coefficient of 43S, is ribonuclease-sensitive, and has a sedimentation profile and base composition similar to the RNA isolated from purified SLE virus. Viral-specific single-stranded RNA isolated from group A arbovirus (9, 22, 28) and that from dengue virus (a group B arbovirus; 30)-infected cells or virions have similar sedimentation profiles, base compositions, and sensitivities to ribonuclease.

The ribonuclease-resistant 20S RNA isolated from SLE virus-infected cells appears to be similar to the 16S to 20S RNA replicative form characteristic of dengue virus (30) and most group A arbovirus infections (9, 10, 17, 21, 27). The 16S RNA which Western equine encephalitis virus makes is reported to be ribonuclease-sensitive (29).

The RNA synthesized by SLE virus includes a 26S species distinct from dengue viral RNA which is virtually devoid of species which sediment at 26S (31) and group A arbovirus 26S RNA, labeled through long exposure to ^{3}H-uridine, which has a base composition similar to single-stranded 40S RNA and is ribonuclease-sensitive (9, 17, 28). The exact nature and function of the 26S form synthesized during replication of SLE virus RNA have not yet been resolved. Our preliminary experiments indicate that SLE virus 26S RNA labeled after 1 hr of exposure to ^{3}H-uridine is digested by ribonuclease (20 μg/ml) to acid-precipitable fragments (*unpublished data*) similar to those of the 26S RNA made by Semliki Forest virus (SFV; 8, 9). Short radioactive-precursor pulse experiments with SLE virus-infected cells revealed radioactivity to be first associated with the 20S form, then the 26S form, and finally the 43S virion precursor RNA. Short-pulse precursor experiments with SFV-infected cells revealed similar results (9, 10), except that the rate of 43S appearance in the SLE virus-infected cells was slower. It is not inconsistent with our data to suggest that the 20S SLE virus and the 26S SLE virus are involved in synthesis of progeny single-stranded RNA. However, it is not yet possible to define their exact function. The 26S RNA formed by SLE virus is distinct from group A virus 26S RNA in its increased resistance to ribonuclease, its base composition which is similar to 20S RNA, and its

slower rate of labeling. Although some similarities do exist between the various types of 26S RNA associated with the replication of group A and group B viral RNA, there are significant differences in the temporal relationships of RNA synthesis to virion formation and in the types of RNA characteristic of SLE virus infection to indicate that there are fundamental differences in the mechanisms by which these viruses multiply.

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