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Membrane-Associated Replication Complex in Arbovirus Infection[1]

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Cytoplasmic extracts of chicken embryo fibroblast cells infected with Semliki Forest virus were subjected to isopycnic centrifugation in discontinuous sucrose gradients. Seven distinct bands were usually formed. The four upper bands contained predominantly smooth membranes and the lowest band was enriched in rough endoplasmic reticulum. One fraction (fraction 5), banding at a density of 1.16 g/cm^3, was found to be heavily enriched in pulse-labeled ribonucleic acid (RNA), viral RNA polymerase, and viral RNA forms associated with RNA replication. Thus, fraction 5 evidently contained a membrane-associated viral replication complex of a type previously defined in picornavirus infections. Fraction 5 was also consistently enriched with unique membranous structures previously observed in intact cells as type 1 cytopathic vacuoles (CPV-1). When the CPV-1 in fraction 5 were isolated from cells briefly incubated with ^{3}H-uridine and ^{3}H-adenosine prior to cell disruption, a large proportion was found to be labeled by high-resolution autoradiography. Thus, ultrastructural, biochemical, and biological evidence were all consistent with the interpretation that the CPV-1 membranes represent a significant element of the viral replication complex.

Studies on the growth of group A arboviruses have indicated that their replicative processes are carried out on cell membranes. Both viral ribonucleic acid (RNA) and protein are synthesized on very rapidly sedimenting structures which can be solubilized by ionic and nonionic detergents (4, 15, 19, 22, 31, 32, 37). These structures were thought to be cell membranes. In addition, the viral RNA polymerase is found in the 13,000 $\times g$ fraction (mitochondrial fraction) of cytoplasmic extracts and is also thought to be membrane-associated (23, 27, 34; J.G. Levin and R.M. Friedman, Fed. Proc. p. 590, 1971).

Electron microscopy of chicken embryo cells infected with Semliki Forest virus (SFV) disclosed the presence of unique membranous structures (1, 15, 21) which were referred to as type 1 cytopathic vacuoles (CPV-1). The accompanying paper by Grimley et al. summarizes ultrastructural evidence that the biogenesis of CPV-1 is related to the replication of group A arboviruses in cells of many types. In an earlier study, by using high-resolution autoradiography (21), it was shown that the CPV-1 in SFV-infected chicken embryo cells were labeled by a short pulse of ^{3}H-uridine during the exponential phase of viral growth. This observation suggested that CPV-1 are loci of viral RNA synthesis.

The object of the present study was to isolate specific membranous fractions from SFV-infected chicken cells and to investigate the role of CPV-1 and other membranes in viral replication. To approach this problem experimentally, cytoplasmic extracts were subjected to isopycnic centrifugation in discontinuous sucrose gradients. This procedure resulted in the separation of seven distinct membrane fractions and a partial purification of CPV-1. The CPV-1 were consistently found only in fraction 5, which was also enriched in pulse-labeled viral RNA, viral RNA polymerase, and the viral RNA forms associated with RNA replication.

MATERIALS AND METHODS

Materials. Actinomycin D was a gift from Merck, Sharp, and Dohme Research Laboratories (Div. of Merck & Co., Inc., Rahway, N.J.). Adenosine-*2,8*-^3H (7 to 15 Ci/mmole), uridine-*5*-^3H (20 to 25 Ci/mmole), L-leucine-*4,5*-^3H (30 to 50 Ci/mmole), and uniformly labeled L-leucine-^{14}C (250 mCi/mmole) were purchased from New England Nuclear Corp. (Boston, Mass.). ^{3}H-guanosine triphosphate (1.0 Ci/mmole) was obtained from Schwarz BioResearch,

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Inc. (Orangeburg, N.Y.). Adenosine triphosphate, uridine triphosphate, and cytidine triphosphate were purchased from General Biochemicals, Inc. (Chagrin Falls, Ohio). Diethyl pyrocarbonate was purchased from Fluka, AG, Switzerland. Phosphoenolpyruvate and pyruvate kinase were obtained from Calbiochem (Los Angeles, Calif.). Recrystallized acrylamide and *N,N'*-methylene bisacrylamide were obtained from Bio-Rad Laboratories (Richmond, Calif.). Cellulose nitrate filters were purchased from the Millipore Corp. (Bedford, Mass.). Ribonuclease-free sucrose was purchased from Mann Research Laboratories (Orangeburg, N.Y.).

Virus and cell culture. The RWGI strain of SFV, originally obtained from J. Sonnabend, The Mt. Sinai Hospital, New York, N.Y., was used. The procedures for the preparation of SFV pools and chicken embryo fibroblast monolayer cultures have been described (18). Gey's medium refers to the basal salt mixture enriched with 0.2% lactalbumin hydrolysate and 0.08% peptone.

Infection procedures. Monolayers of chicken embryo fibroblasts (ca. 2.5×10^7 cells per 100-mm Falcon Plastic petri dish) were infected with 0.5 ml of SFV at a multiplicity of 20 to 40 plaque-forming units per cell in the presence of 0.5 μg of actinomycin D. In general, between 5.0 and 7.5×10^8 cells were used in an individual experiment. After adsorption for 1 hr at 37 C, 4.5 ml of warm Eagle's medium was added. Incubation was continued at 37 C.

Procedures for labeling viral RNA. At 4.5 hr post-infection, the cells were incubated with fresh Eagle's medium containing ^{3}H-adenosine and ^{3}H-uridine (50 μCi/ml each) for the times specified in the tables. The cells were harvested and fractionated as described later. The buffers and sucrose solutions used for the fractionation each contained nonradioactive adenosine and uridine at 100 times the concentration of the radioactive materials.

Procedures for labeling viral protein. Monolayers of chicken embryo fibroblasts were treated with actinomycin D (2 μg/ml) for 2 hr at 37 C. The drug was removed, and the cells were infected with SFV. After incubation for an additional hour at 37 C, the cells were left for 16 hr at 4 C in Gey's medium containing 10% fetal bovine serum. Fresh Eagle's medium was then added, and the cells were warmed to 37 C. After 4.5 hr, the cells were washed several times with medium lacking amino acids and were then incubated for another hour at 37 C with ^{3}H-leucine (10 μCi/ml) in otherwise leucine-free Eagle's medium. Procedures for harvesting the cells and for fractionation in a discontinuous sucrose gradient are described below.

Cell fractionation. At the times specified in the tables or figures, the infected monolayers were washed five times with ice-cold phosphate-buffered saline (12). The cells were then scraped with a no. 6 rubber stopper into a small volume of saline and sedimented for 10 min at $200 \times g$. The cell pellet was suspended in 5 to 7 ml of reticulocyte standard buffer (RSB) (10 mM tris(hydroxymethyl)aminomethane [Tris], *p*H 7.4, 10 mM NaCl, and 1.5 mM $MgCl_2$), allowed to swell for 15 min at 4 C, and disrupted with 20 strokes in a tight-fitting, glass Dounce homogenizer. Nuclei and cell debris were removed by centrifugation for 10 min at $800 \times g$. The supernatant cytoplasmic extract was then processed by a procedure based on the method of Caliguiri and Tamm (7, 8).

As shown in Fig. 1, the cytoplasmic extract was made 30% (w/w) in sucrose. The extract was then added above the 40% sucrose layer in a discontinuous gradient which had been prepared by layering sucrose-RSB (w/w) solutions in the following order: 3 ml of 65%; 7 ml of 45%; 7 ml of 40%; and 8 to 10 ml of the sample containing 30% sucrose; 7 ml of 25% sucrose, and 3 ml of RSB. The gradients were centrifuged for 17 to 19 hr at $96,000 \times g$ in a Spinco SW27 rotor.

This procedure was subsequently modified in order to obtain membrane fractions which were free of viral nucleocapsids (cores). In the modified procedure, the cytoplasmic extract was first layered over a mixture containing 12 ml of 15% (w/v) sucrose-RSB on top, 12 ml of 30% (w/v) sucrose-RSB in the middle, and 3 ml of 65% (w/w) sucrose-RSB as a cushion. This mixture was then sedimented at $40,000 \times g$ for 30 min in a Spinco SW25 rotor. A highly viscous band (about 2 ml) formed between the 65% sucrose cushion and the 30% sucrose solution. The clear supernatant fluid, containing the viral cores, was removed by aspiration and discarded. The banded material was withdrawn with a Pasteur pipette, diluted to 5 ml with RSB, and evenly dispersed in a Dounce homogenizer. The resulting homogeneous solution was then made 30% (w/w) in sucrose and was appropriately layered in a discontinuous sucrose gradient (Fig. 1).

In some experiments (Tables 6 and 7) a rapid procedure was employed in which a $13,000 \times g$ pellet

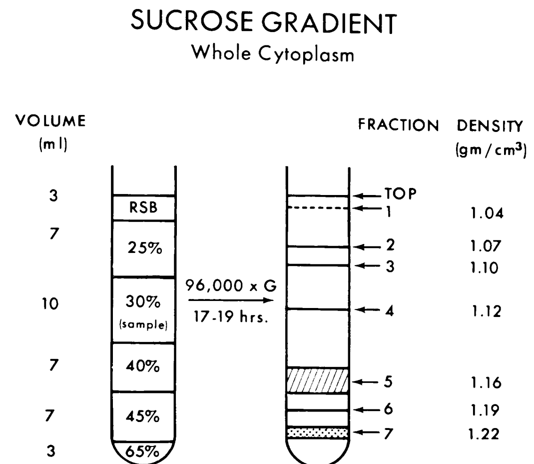

FIG. 1. *Schematic diagram of procedure for fractionation of cytoplasmic membranes by isopycnic centrifugation in a discontinuous sucrose gradient. The position of the seven fractions at equilibrium and representative densities are shown. Further details are given in the text under Materials and Methods.*

("mitochondrial fraction") was made 30% (w/w) in sucrose and then directly applied to the six-layer discontinuous 0 to 65% sucrose gradient (Fig. 1). It should be stressed that the biochemical findings were the same regardless of the manner in which nucleocapsids had been removed.

After overnight sedimentation in the discontinuous gradient, membrane fractions were visible as white, turbid bands of varying width. Seven fractions usually resulted when the procedure shown in Fig. 1 was employed. The modified procedures (cores removed) always yielded four fractions designated as 5, 6A, 6B, and 7; the upper four fractions, obtained by the original procedure (Fig. 1) containing smooth membranes of low densities, were no longer present.

The fractions were collected with a Pasteur pipette. The densities of the fractions were determined by weighing 100 μliters of the sample and dividing this value by the weight of a nominal 100 μliters of water delivered with the same pipette. Representative densities for the various fractions are shown in Fig. 1 and Table 1. Where indicated, the fractions were freed of sucrose by resedimentation for 2 hr at high speeds (generally 100,000 × g), and the resulting pellets were then concentrated in a small volume of buffer.

Radioactivity was determined by precipitating a portion of each fraction (generally 1/100 of the volume) with 1 ml of cold 5% trichloroacetic acid (determination of RNA) or 10% trichloroacetic acid (determination of protein). The precipitates were collected on type HA cellulose nitrate filters (25-mm diameter, 0.45 μm pore size) and washed five times with 3-ml portions of cold 5% trichloroacetic acid. The filters were counted in 10 ml of toluene-Liquifluor in a liquid scintillation spectrometer (Packard Instrument Co., Inc.). The data reported in the tables represent total radioactivity of each fraction.

Preparation of membrane fractions for determination of SFV RNA polymerase activity. Chicken embryo fibroblast cells were infected with SFV as described above. At 4 hr postinfection, the cells were incubated for an additional 1.5 hr with fresh Eagle's medium containing actinomycin D at 2 μg/ml. The cells were then harvested, and the 13,000 × g pellet obtained

TABLE 1. *Summary of ultrastructural observations*

Fraction no.	Density (g/cm³)	Electron microscopy[a]
1	1.04	Smooth membranes (small vesicles)
2	1.07	Smooth membranes (small vesicles)
3	1.10	Smooth membranes (mixed vesicles)
4	1.12	Smooth membranes (large vesicles)
5	1.16	CPV-1 (smooth membranes, mitochondrial remnants)
6	1.19	Ribosomes (very rare CPV-1)
7	1.21	Granular endoplasmic reticulum (viral nucleocapsids [CPV-2], virions)

[a] CPV-1, type 1 cytopathic vacuoles; CPV-2, type 2 cytopathic vacuoles.

from the cytoplasmic extract was fractionated in a discontinuous sucrose gradient as described previously. All buffers and sucrose solutions contained 10 mM β-mercaptoethanol. After resedimentation of the membrane fractions, the resulting pellets were each resuspended in 0.2 ml of 10 mM Tris buffer, pH 7.4, containing 1 mM $MgCl_2$ and 10 mM β-mercaptoethanol. Portions were removed from each fraction for determination of protein concentration (24) and RNA polymerase activity. The enzyme assay was performed in 0.1-ml reactions containing 50 mM Tris buffer (pH 8.0), 10 mM $MgCl_2$, 0.025 μg of actinomycin D, 1.75 μmoles of β-mercaptoethanol, 10 mM phosphoenolpyruvate, 4 μg of pyruvate kinase, 10 nmoles each of adenosine triphosphate, uridine triphosphate, cytidine triphosphate, and ^{3}H-guanosine triphosphate (2.7 × 10^6 counts/min), and 20 μliters of the specified membrane fraction. Reactions were incubated for 30 min at 37 C and were terminated by the addition of cold 5% trichloroacetic acid containing 0.125 M sodium pyrophosphate. After 5 to 10 min at 4 C, the precipitated RNA was collected on cellulose nitrate filters and washed 10 times with 3-ml portions of cold 5% trichloroacetic acid. The filters were counted in a liquid scintillation spectrometer, as described previously. All reactions were carried out in duplicate, and the results shown in Table 7 have been corrected for radioactivity (537 counts/min) obtained on the filter from a standard reaction without enzyme.

Preparation of viral nucleocapsids. Chicken embryo fibroblast cells were infected with SFV and, at 4 hr postinfection, fresh Eagle's medium containing ^{3}H-adenosine and ^{3}H-uridine (50 μCi/ml each) was added. Incubation at 37 C was continued for another hour. A cytoplasmic extract was prepared according to the procedures given previously. A 0.3-ml sample of the extract was layered on a 4.4-ml, 15 to 30% (w/v) linear sucrose gradient prepared in RSB. Centrifugation was for 1 hr at 100,000 × g in a Spinco SW50 rotor. After collecting 32 fractions (0.15 ml each), 5-μliter portions of each fraction were spotted on Whatman no. 2 filter-paper discs and precipitated with 1.7% perchloric acid as previously described (15). Two of the fractions (fractions 8 and 9) contained in the peak sedimenting at 140S (15) were combined and used as the source of labeled viral cores for the experiment shown in Table 5.

RNA extraction and analysis. Fractions were collected and resedimented as described previously. The membrane pellets were resuspended with a Teflon homogenizer in 1 ml of buffer containing 10 mM Tris (pH 7.2), 0.1 M NaCl, and 1 mM ethylenediaminetetraacetate, and were then shaken with 30 μliters of diethyl pyrocarbonate (29) on a Vortex mixer. The vigorous mixing was continued for a few minutes after addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.5%. Viral RNA was isolated by performing two extractions with phenol at room temperature, as previously described (16). The procedures used for analysis of viral RNA by polyacrylamide gel electrophoresis are given elsewhere (23).

Gel electrophoresis of viral proteins. Fractions were collected and resedimented as described previously. Membrane pellets to be analyzed by gel electrophoresis

were resuspended with a Teflon homogenizer in 0.5 ml of a solution containing 1% β-mercaptoethanol, 1% SDS, and 10% glacial acetic acid, and were incubated for 1 hr at 37 C. The solution was then dialyzed overnight against 2 liters of buffer containing 0.01 M sodium phosphate (pH 7.1), 0.1% SDS, and 0.1% β-mercaptoethanol. The ^{14}C-labeled marker from the whole cytoplasm of SFV-infected cells was prepared as follows. Chicken embryo fibroblast cells were treated with actinomycin D and infected with SFV as described previously and, at 5 hr postinfection, the cells were washed with Eagle's medium lacking leucine and then incubated for 1 hr at 37 C with ^{14}C-leucine (0.5 μCi/ml) in otherwise leucine-free Eagle's medium. The cells were harvested in the usual manner and then resuspended in 1 ml of RSB containing 0.5% Nonidet P-40; the nuclei were removed by centrifugation for 10 min at 800 × g, and the supernatant was made 1% in β-mercaptoethanol, 1% in SDS, and 10% in glacial acetic acid. Incubation for 1 hr at 37 C was followed by overnight dialysis of the sample against the phosphate buffer described above.

Electrophoresis, on 10% polyacrylamide gels 7 cm in length, was performed according to the method of Maizel (26). The gels were prerun for 30 min at room temperature at a constant current of 5 ma per tube to remove excess catalyst. The samples contained a few drops of a tracking dye, 0.005% bromphenol blue (pH 7), and were run at 5 ma per gel until the blue color of the tracking dye was no longer visible, i.e., for about 7 hr. After electrophoresis, the gels were transferred to test tubes containing 20% trichloroacetic acid and left for 1 hr at room temperature. The gels were washed several times with cold 5% trichloroacetic acid and were then sliced, solubilized, and counted as previously described (23).

Electron microscopy. Fraction samples collected with a Pasteur pipette were diluted with RSB and centrifuged at 100,000 × g in a Spinco type 50 rotor for 2 hr to form pellets. The pellets thus obtained were fixed in 3% glutaraldehyde or 2% paraformaldehyde in 0.1 M Sorensen's buffer at pH 7.4. After 1 to 2 hr of fixation, the pellets were washed in buffer and postfixed in phosphate-buffered 1% OsO_4. The pellets were then dehydrated in ethanol and embedded in epoxy resin (25). Ultrathin sections obtained with an automatic ultratome were double-stained with 10% uranyl acetate in methanol and lead citrate (36) prior to examination in an Hitachi 11E electron microscope at original magnifications of 10,000 to 24,000 in diameter.

High-resolution autoradiography with 1,000-nm sections was performed according to procedures detailed previously (21) by using the basic techniques of Caro (9, 10).

RESULTS

Separation of membrane fractions and characterization by electron microscopy. Previously it was shown that the cytoplasmic membranes of uninfected (5) and poliovirus-infected (7, 8) HeLa cells could be distinguished as separate bands after isopycnic centrifugation of extracts in a discontinuous sucrose gradient. To determine whether a similar fractionation scheme would be useful for studies on SFV replication, cytoplasmic extracts of SFV-infected cells were prepared and sedimented overnight in a discontinuous gradient as illustrated in Fig. 1. Seven membrane fractions, visible as turbid bands, were collected and resedimented, and the resulting pellets were prepared for examination in the electron microscope. A summary of the findings is given in Table 1.

Fractions 1 to 4 were found to contain smooth vesicular membranes of graded sizes, and it may be seen, for example, that much larger vesicles were present in fraction 4 than in fraction 1 (Fig. 2). Fraction 6 consisted of an almost pure preparation of membrane-free ribosomes, whereas fraction 7 contained a mixture of rough endoplasmic reticulum, viral nucleocapsids and CPV-2 (17, 21), and some viral particles (Fig. 3).

The contents of fraction 5 were of greatest interest. In repeated experiments, this band contained a mixture of smooth membranes, mitochondrial fragments, and intact CPV-1 (Fig. 4). In sections of the pelleted fraction, up to four CPV-1 were identified per grid square. CPV-1 in thin sections of whole cells are illustrated in the accompanying paper by Grimley et al.

Localization of viral RNA. Electron microscopy demonstrated that well-defined membrane fractions from cytoplasmic extracts of chicken cells can be separated by using the procedure of Fig. 1. Because it had been found that in poliovirus-infected HeLa cells viral RNA synthesis is associated with a particular membranous fraction (7), it was of interest to determine if this was true for SFV-infected chicken cells.

In the experiment reported in Table 2, SFV-infected cells were incubated for 5 min with Eagle's medium containing ^{3}H-uridine and ^{3}H-adenosine. The cells were processed as described in Materials and Methods and in Fig. 1, and for each of the seven fractions both the density and total radioactivity were determined. As may be seen (Table 2), fraction 5, with a density of 1.159 g/cm^3, contained at least twice as much radioactivity as any of the other bands. The enrichment of fraction 5 in pulse-labeled RNA was consistent.

In some studies, pulse-labeled fractions were also examined in the electron microscope. In several experiments, when high-resolution autoradiography was performed on thin sections of fraction 5, grains were frequently localized above intact CPV-1 (Fig. 5). After 4 weeks of exposure, over 75% of a total of 40 CPV-1 counted in one experiment were labeled. Some grains were also found over disrupted smooth membranes and

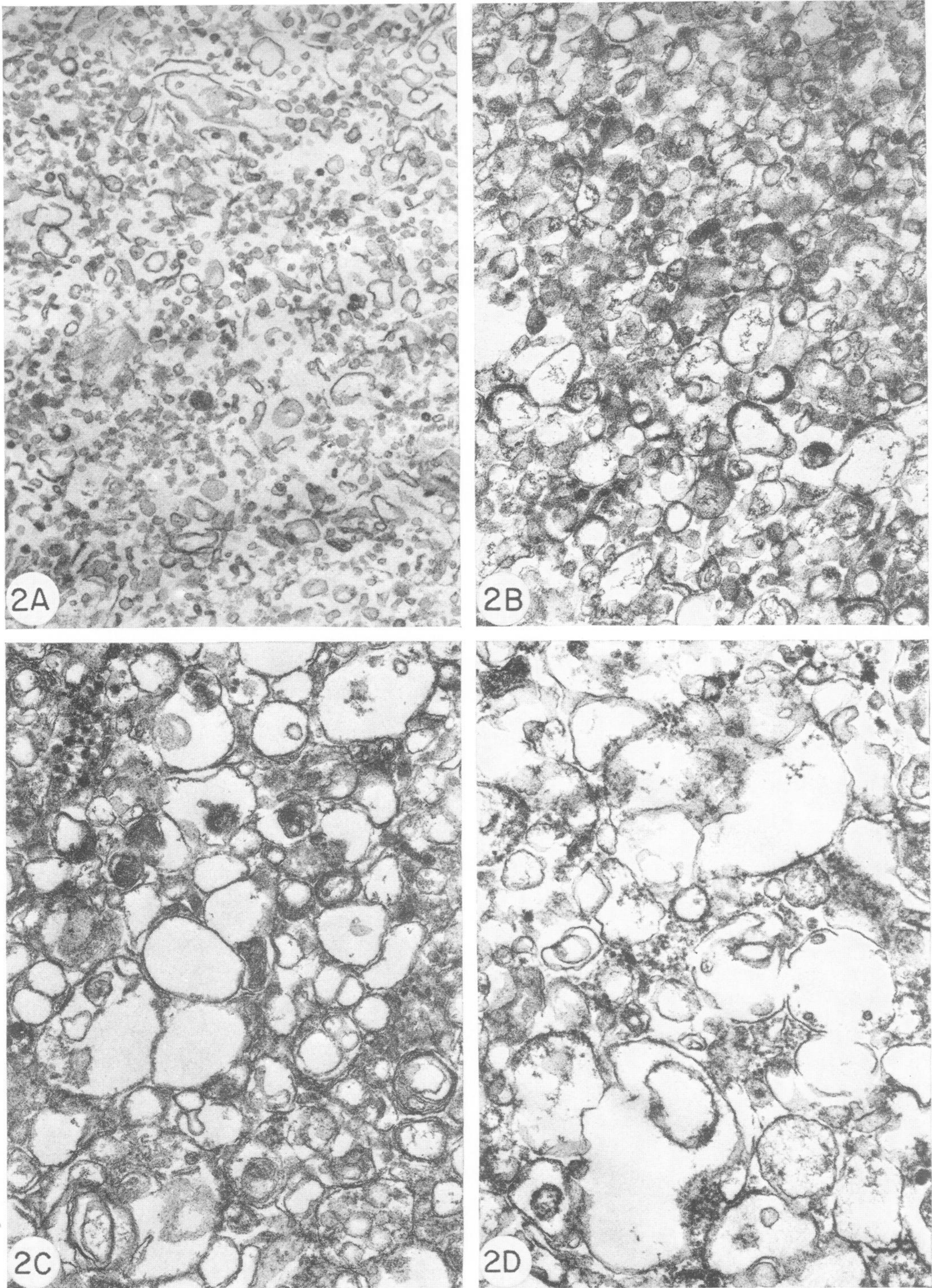

FIG. 2. *Thin sections of low-density fractions (1 to 4) obtained from Semliki Forest virus-infected chicken embryo cell extracts after isopycnic sedimentation in a discontinuous sucrose gradient according to the procedure shown in Fig. 1. 2A, Fraction 1; 2B, fraction 2; 2C, fraction 3; 2D, fraction 4. Vesicular membranes have separated into classes of graded sizes.* ×*32,500.*

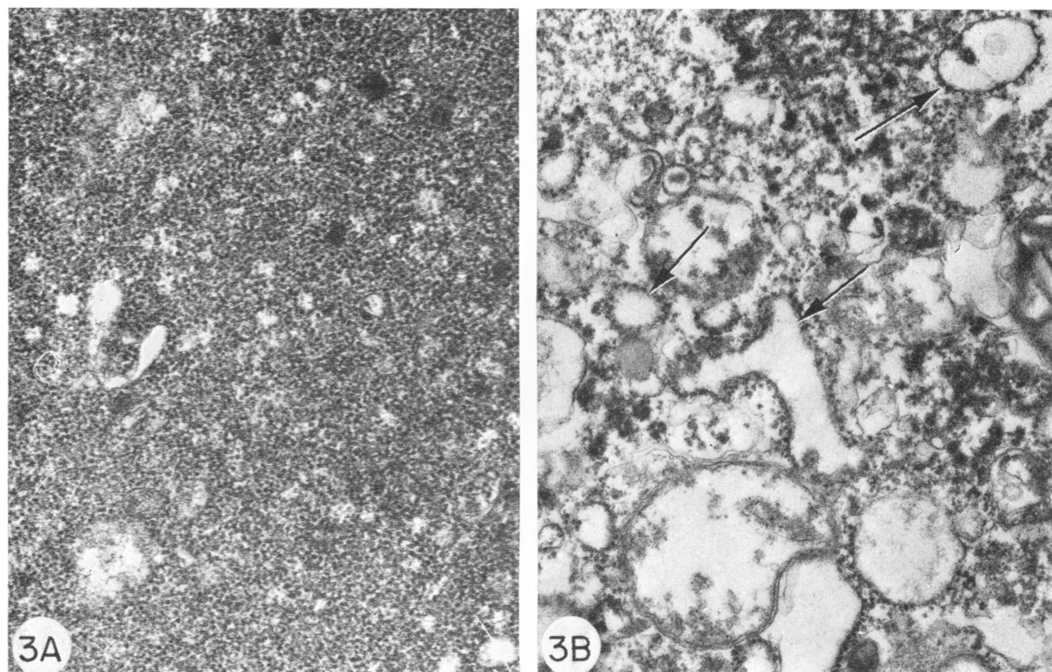

FIG. 3. *Thin sections of high-density cytoplasmic fractions 6 and 7 obtained according to the procedure shown in Fig. 1. Fraction 6 consisted almost entirely of membrane-free ribosomes (3A). Fraction 7 contained numerous elements of ribosome-bound endoplasmic reticulum (arrows, 3B). ×32,500.*

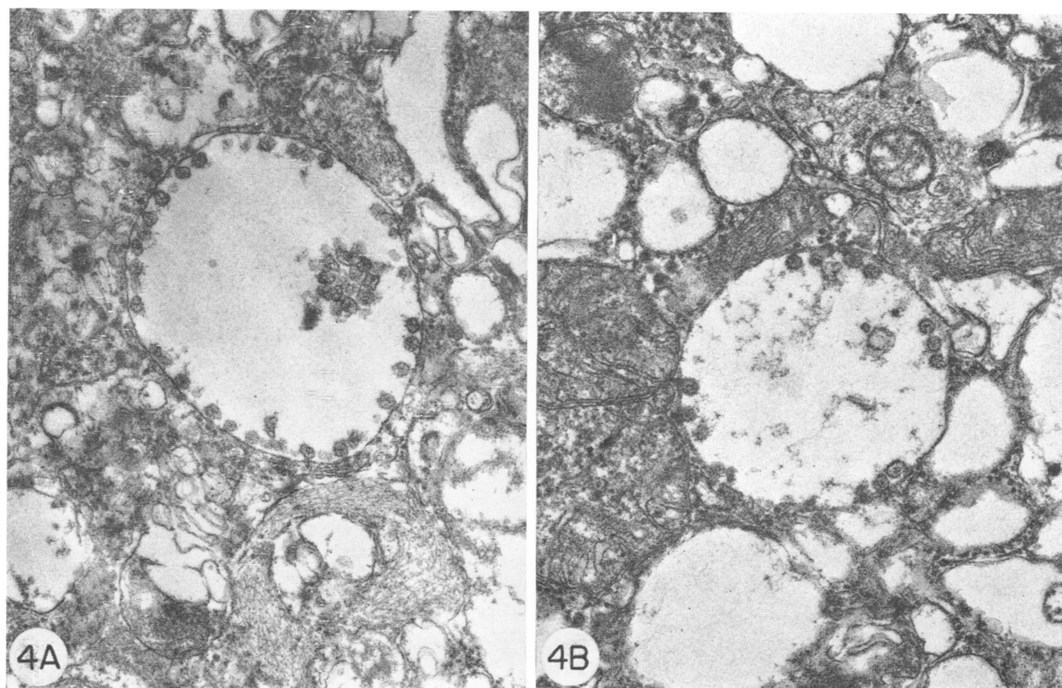

FIG. 4. *Thin sections of fraction 5 (density 1.16 g/cm³) obtained according to the procedure shown in Fig. 1. ×32,500.*

TABLE 2. *Distribution of ^{3}H-labeled Semliki Forest virus RNA in membrane fractions after a 5-min pulse*

Fraction	Density (g/cm^3)	Radioactivity (counts/min)
1	1.051	16,200
2	1.075	101,250
3	1.112	196,600
4	1.124	193,050
5	1.159	719,250
6	1.195	351,000
7	1.240	157,050

over clusters of free ribosomes. The close packing of membranous materials in the pellets precluded any quantitative analysis of the findings, but repeated observations suggested that intact CPV-1 were more heavily labeled than other elements of fraction 5. The autoradiography thus correlated with the data of Table 2 and demonstrated that the membranes of fraction 5, including CPV-1, are associated with the synthesis of SFV RNA.

Previous studies on SFV (21) and poliovirus (7, 8) have suggested that viral RNA synthesis at one site is followed by migration of RNA to another site where protein synthesis and assembly take place. Therefore, it was of interest to note that incubation of SFV-infected cells with RNA precursors for 1 hr, between 5 and 6 hr after infection (Table 3), resulted in a different distribution of radioactivity than that obtained with a short labeling period (Table 2). With a 1-hr incubation, fraction 7 contained the most radioactivity.

Localization of viral protein. By 4 hr postinfection, over 90% of cellular protein synthesis is virus directed (14, 35). To determine the distribution of viral proteins in the various membrane fractions, infected cells were incubated with ^{3}H-leucine for 1 hr between 4.5 and 5.5 hr postinfection. As shown in Table 4, most of the radioactivity was found in fraction 7; about half as many counts were found in fraction 6. Short labeling periods yielded qualitatively similar results; i.e., the largest proportion of radioactivity was contained in fraction 7. The latter data are in accord with the observation that rough endoplasmic reticulum is a dominant feature of fraction 7 (Fig. 3).

As mentioned previously, fraction 7 also contained viral nucleocapsids (Fig. 3). To estimate the distribution of cores more quantitatively, partially purified ^{3}H-labeled core particles were added to a cytoplasmic extract prepared from unlabeled cells, and the mixture was fractionated according to the procedure in Fig. 1. As shown in Table 5, nine bands were observed. In this particular experiment, fractions 6 and 7 were each separated into two subfractions designated as 6A and 6B and 7A and 7B, respectively. As anticipated from electron microscopy (Table 1), most of the radioactivity was found in fraction 7.

The banding of viral core particles in fraction 7 requires that the results of Tables 3 and 4 be interpreted with caution, because it is known that, as with polyribosomes, radioactive amino acids are very rapidly incorporated into SFV nucleocapsids (4, 13). Thus, core particles are labeled during a 20-sec to 1-min pulse with radioactive amino acids and, with longer incubation periods, become heavily labeled. Indeed, when long incubations with RNA precursors are carried out, the radioactivity found in cytoplasmic extracts is also associated predominantly with core structures. Consequently, in interpreting the studies on fraction 7, it was necessary to distinguish between labeling of viral membrane structures and of nucleocapsids.

Results after removal of nucleocapsids. To resolve the problem posed by the accumulation of nucleocapsids in fraction 7, the fractionation procedure was modified so that cores would be eliminated from the cytoplasmic extract. SFV nucleocapsids have a sedimentation coefficient of 140*S* and are pelleted only after high-speed centrifugation for an hour or more (2, 15). Thus, to remove these particles, cytoplasmic extracts were sedimented for relatively short periods of time; the resulting supernatant fluid was discarded and the membranous pellet was analyzed in a discontinuous sucrose gradient (Fig. 1). The findings, to be presented below, were unaffected by minor variations in the conditions used to prepare the membranous pellet (see Materials and Methods).

When the revised procedure was employed, bands corresponding to fractions 1 to 4 of the original procedure were no longer evident, but four membrane fractions designated as 5, 6A, 6B, and 7 were detectable (Table 6). These fractions corresponded in density to the previously observed bands 5, 6, and 7. Examination in an electron microscope showed that band 5 contained CPV-1, mitochondrial fragments, and smooth membranes; band 7 contained rough endoplasmic reticulum, but no viral cores. Band 6A was similar to band 5, and band 6B was similar to band 7.

Fractionation of infected cells which had been incubated for 3 min with ^{3}H-adenosine and ^{3}H-uridine resulted in a 6- to 8-fold enrichment of radioactivity in fraction 5, compared with fractions 6A and 6B (Table 6), in accord with the data of Table 2. Incubation with radioactive RNA precursors for 1 hr, however, led to a lower

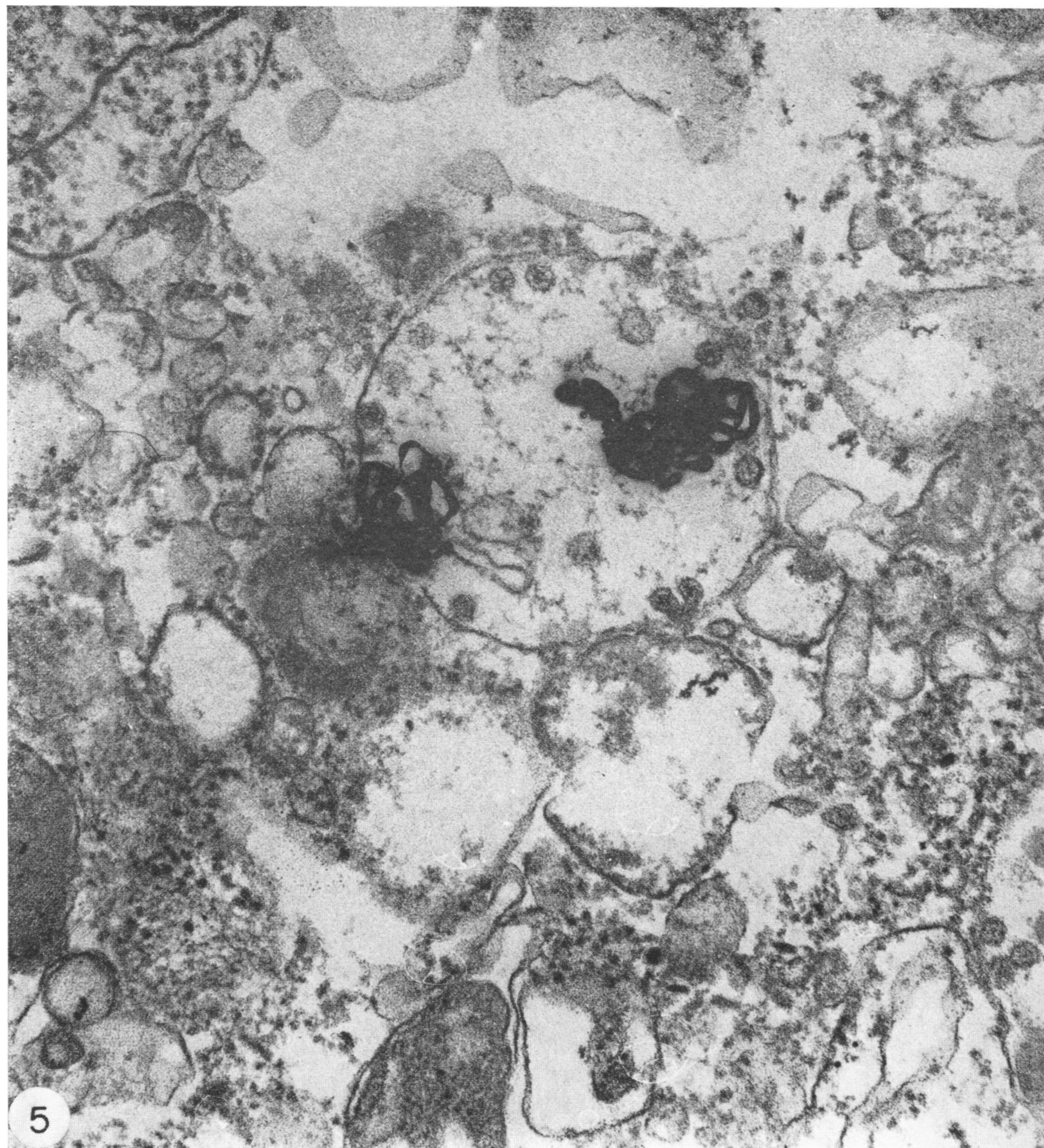

FIG. 5. *Autoradiograph of a thin section of fraction 5 (see Fig. 4), demonstrating developed silver grains over type 1 cytopathic vacuoles. Before disruption and fractionation, the chicken embryo cells had been pulsed for 5 min ^{3}H-uridine and ^{3}H-adenosine at 5 hr after infection with Semliki Forest virus (see Materials and Methods). ×60,000.*

amount of radioactivity in fraction 7 than was seen in the experiment of Table 3. Nevertheless, even though core particles were no longer present, fraction 7 contained more counts than any of the other fractions, including fraction 5.

As pointed out above, both polyribosomes and viral nucleocapsids become rapidly labeled when cells are given brief pulses with radioactive amino acids. Thus, the methods which effectively eliminate core particles from the cytoplasmic extract should be useful for studies on virus protein synthesis. In several experiments employing this technique, fraction 7 contained the most radioactivity following a 1- or 2-min incubation with ^{3}H-leucine. Occasionally, however, fractions 5 and 6 were also heavily labeled. The explanation for the disparity in these findings is under investigation. Nevertheless, the results taken together do

TABLE 3. *Distribution of ^{3}H-labeled Semliki Forest virus RNA in membrane fractions after a 1-hr pulse*

Fraction	Radioactivity (counts/min)
1	3,610
2	36,140
3	82,030
4	124,860
5	546,090
6	840,210
7	1,568,200

TABLE 4. *Distribution of ^{3}H-labeled Semliki Forest virus protein in membrane fractions after a 1-hr pulse*

Fraction[a]	Radioactivity (counts/min)
1	7,550
2	39,350
3	58,900
4	77,000
5	270,200
6	358,600
7	689,400

[a] The seven membrane fractions obtained by isopycnic centrifugation were diluted with reticulocyte standard buffer (RSB) and sedimented at 100,000 × *g* for 2 hr in a Spinco type 50 fixed-angle rotor. The resulting pellets were each resuspended with a Teflon homogenizer in 1 ml of RSB.

suggest that rough endoplasmic reticulum in fraction 7 is the site of SFV protein synthesis (17).

Localization of SFV RNA polymerase in nucleocapsid-free fractions. The viral RNA-dependent RNA polymerase elaborated during SFV infection is a membrane-associated enzyme which catalyzes the formation of the double-stranded ribonuclease-resistant replicative forms (23, 27). To determine which of the membrane fractions contained the enzyme, a cytoplasmic extract from which cores had been removed was fractionated in a discontinuous sucrose gradient, and the resulting four fractions were assayed for polymerase activity. As shown in Table 7, most of the activity was found in fraction 5, with smaller amounts also present in fractions 6A and 6B. Fraction 7 consistently had very low levels of enzyme activity. Similar results (i.e., highest enzyme activity in fraction 5) were obtained when the seven membrane fractions from a cytoplasmic extract prepared without removal of core particles were assayed for polymerase activity. These observations again pointed to fraction 5 as a site for viral RNA synthesis.

Characterization of viral RNA and proteins in fraction 5. Several viral RNA species have been identified in cells infected with SFV (11, 18, 23, 30) and other group A arboviruses (33). To determine whether there was an enrichment of any particular viral RNA forms in fraction 5, the RNA species were extracted and analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 6, the high-molecular-weight RNA species thought to be associated with RNA replication, i.e., the replicative intermediate and the replicative forms, were present in higher concentration in fraction 5 than in the ^{32}P-labeled SFV marker RNA from whole cells.

It was of further interest to determine which virus-specific proteins were associated with fraction 5. Accordingly, ^{3}H-labeled proteins from

TABLE 5. *Distribution of ^{3}H-labeled Semliki Forest virus (SFV) nucleocapsids in membrane fractions*[a]

Fraction[b]	Density (g/cm^{3})	Radioactivity (counts/min)
1	1.038	280
2	1.064	560
3	1.100	2,210
4	1.116	640
5	1.160	1,630
6A	1.174	3,030
6B	1.192	9,260
7A	1.204	16,230
7B	1.229	45,510

[a] An unlabeled cytoplasmic extract was prepared from infected cells harvested at 4.5 hr postinfection. To this extract was added approximately 0.3 ml of ^{3}H-labeled SFV nucleocapsids (270,000 counts/min) which had been isolated as described in Materials and Methods.

[b] In this particular experiment, fractions 6 and 7 separated into two subfractions designated as 6A and 6B and 7A and 7B, respectively.

TABLE 6. *Distribution of ^{3}H-labeled Semliki Forest virus RNA in membrane fractions after a 3-min pulse*

Fraction[a]	Density (g/cm^{3})	Radioactivity (counts/min)
5	1.159	649,600
6A	1.164	96,800
6B	1.194	82,600
7	1.217	25,400

[a] Viral nucleocapsids were removed from the cytoplasmic extract by sedimentation of the extract at 13,000 × *g*. The 13,000 × *g* pellet was resuspended in reticulocyte standard buffer and fractionated in a discontinuous sucrose gradient as described in Materials and Methods.

TABLE 7. *Distribution of Semliki Forest virus RNA polymerase activity in membrane fractions*

Fraction[a]	Density (g/cm³)	³H-guanosine monophosphate Incorporated[b] (counts/min)	Protein[b] (µg)	Specific activity (counts/min per mg of protein)
5	1.161	10,541	118	89,300
6A	1.175	979	26	37,700
6B	1.193	3,432	108	31,800
7	1.201	912	93	9,800

[a] The procedure used to remove viral nucleocapsids from the cytoplasmic extract prior to fractionation in a discontinuous sucrose gradient is given in Materials and Methods.

[b] The values given are for a 0.1-ml polymerase reaction. The total amount of protein present in each fraction can be calculated by multiplying the values in column 4 by a factor of 10.

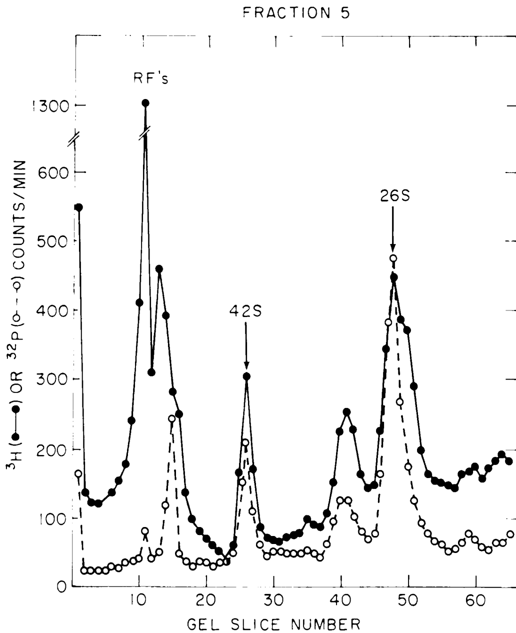

FIG. 6. *Polyacrylamide gel electrophoresis of ³H-labeled Semliki Forest virus (SFV) RNA species found in fraction 5. At 5 hr postinfection, the cells were incubated for 1 hr with fresh Eagle's medium containing ³H-adenosine and ³H-uridine (75 µCi/ml each). The cells were harvested and fractionated according to the modified procedure (viral core particles removed) given in Materials and Methods. The RNA present in fraction 5 was extracted as described. A sample containing 50 µliters of ³H-labeled SFV RNA from fraction 5 and 8 µliters of ³²P-labeled SFV RNA from unfractionated chicken embryo cells (23) was subjected to electrophoresis for 4 hr on a composite 2.0% polyacrylamide-0.5% agarose gel, as previously described (23). The ³H counts have been corrected for 5% crossover of ³²P into the ³H channel. ●—●, ³H; ○- -○, ³²P.*

fraction 5 were analyzed by polyacrylamide gel electrophoresis using ¹⁴C-labeled proteins from a crude cytoplasmic extract as a marker. The resulting electrophoretic pattern was similar to that previously reported from this laboratory for group A arbovirus-infected cells (14), and at least five or six major peaks could be clearly identified (Fig. 7). Two of these peaks were structural elements of the virus, the membrane glycoprotein and core protein. Because the viral cores had been removed in this preparation, the sample was relatively deficient in core protein; however, none of the other proteins appeared to be particularly enriched.

DISCUSSION

The replication complex of an animal virus is defined as a structure containing the template for viral replication, replicating strands of viral RNA (replicative intermediate), and the viral RNA polymerase (3, 20, 28). In the present experiments with discontinuous sucrose gradients, all of these elements banded with membranes in fraction 5 (density of 1.16 g/cm³). Arbovirus-specific membranous structures referred to as CPV-1 also banded in this fraction.

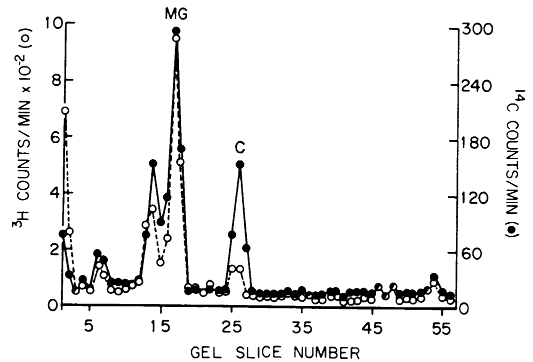

FIG. 7. *Polyacrylamide gel electrophoresis of ³H-labeled Semliki Forest virus (SFV) proteins found in fraction 5. Chicken embryo fibroblast cells were treated as described in Materials and Methods except that the amount of ³H-leucine used to label the cells was 50 µCi/ml. Procedures for preparation of a cytoplasmic extract with viral core particles removed and for fractionation in a discontinuous sucrose gradient are given in Materials and Methods. Fraction 5 was prepared for gel electrophoresis as described. A sample containing 100 µliters of the ³H-labeled SFV proteins of fraction 5 and 10 µliters of a marker ¹⁴C-labeled preparation from the whole cytoplasm of SFV-infected chicken embryo cells was subjected to electrophoresis on a 10% polyacrylamide gel by using the procedures given in Materials and Methods. The gel slices were counted with settings which eliminated crossover of ¹⁴C into the ³H channel. MG refers to membrane glycoprotein and C to core protein; ○- -○, ³H; ●—●, ¹⁴C.*

It is interesting to compare the results we have presented with those reported for poliovirus. Replication complexes for poliovirus have been studied in great detail (3, 20, 28) and provide a precedent for the present work. In poliovirus infection the replication complex contains the replicative intermediate and the viral polymerase, but no ribosomes. The poliovirus replication complex has also been shown to be membrane-associated, banding in isopycnic sucrose gradients at a density of 1.119 g/cm^3 (6, 7). Some pulse-labeled viral RNA and viral polymerase were also found at a density of 1.179 g/cm^3 (7). All of these fractions contained smooth membranes, but lacked any ultrastructure which was clearly shown to be virus-specific. In the case of group A arboviruses, ultrastructurally distinctive membrane structures (CPV-1) appear to be specific sites, or at least one of several sites, of viral RNA replication. Thus, the most interesting question raised by the present experiments is whether the SFV replication complex found in membrane fraction 5 is associated with the CPV-1 by more than hydrodynamic factors. Although the CPV-1 could be only partially purified in the sucrose gradients, autoradiograms provided direct qualitative evidence for an association of some RNA replication with the CPV-1 membranes. Other biological, ultrastructural, and biochemical evidence presented here and in the accompanying paper by Grimley et al. are also consistent with the proposal that the membranes of CPV-1 (possibly the enclosed spherules) represent a significant element of the arbovirus replication complex.

Poliovirus proteins appear to be synthesized on membranous structures which band at a density of 1.256 g/cm^3 (7). Our data suggest that fraction 7 with a similar density (1.21 g/cm^3) is a specific site of SFV protein synthesis. Thus, fraction 7 contains the rough, endoplasmic reticulum and was found to accumulate viral RNA labeled during a 1 hr exposure of cells to ^{3}H-uridine and ^{3}H-adenosine as well as viral proteins labeled during short and long exposures to radioactive amino acids. In addition to membrane-bound ribosomes, viral nucleocapsids were also identified in fraction 7. This was an interesting correlation because nucleocapsids were previously shown to incorporate radioactive nucleic acid precursors and amino acids with kinetics similar to those observed for labeling of fraction 7 (4, 13). Furthermore, formation of nucleocapsids on the rough, endoplasmic reticulum has been suggested by electron microscopy (17).

In the experiments in which viral cores were removed from the cytoplasmic extracts, viral RNA was still present in fraction 7. This membrane-associated RNA could be the viral messenger form, a species not yet identified in SFV infection. Although further studies have been complicated by high levels of ribonucleolytic activity, we are attempting to examine the viral RNA species remaining in fraction 7 after removal of nucleocapsids.

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