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Inhibition of Arbovirus Assembly by Cycloheximide

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Addition of cycloheximide (100 μg/ml) to cultures of chick cells infected with Semliki Forest virus (SFV) halted subsequent increase in virus titers. When added after 4 hr of infection, the drug had no effect on the rate of viral ribonucleic acid (RNA) synthesis, although marked inhibition of protein synthesis was seen. All of the previously identified forms of SFV RNA were seen in the drug-treated cells at higher concentrations than were present in untreated controls. The latter observation appeared to result from a failure to form viral "cores" or nucleocapsids in the cycloheximide-treated cells, resulting in sequestration of viral RNA intracellularly. The failure to form new virus cores was correlated with the failure of type II cytopathic vacuoles to appear in thin sections. Virus budding from the cell surface and the formation of type I cytopathic vacuoles persisted in cycloheximide-treated cells. The cellular pool of the major protein present in the virus core appeared to be small. None of this protein was found in a free pool in cytoplasm. The results indicated that, in the presence of cycloheximide, virus assembly was impaired because of the small size of the cellular pool of the major protein required for virus core formation.

Formation of a nucleocapsid or core by arboviruses is a step in the assembly of the virions of this group (7). The core structure contains the ribonucleic acid (RNA) of the virion (7, 13) and either a single protein (14) or two proteins, a major and a minor component (6). Although newly synthesized viral RNA takes up to 30 min to become associated with the core (7), viral protein is rapidly integrated into the structure (2, 4) and some counts may be seen in the core within 30 sec after a pulse of radioactive amino acids (Friedman, *unpublished data*).

The formation of the cores of group A arboviruses appears to take place on cytoplasmic vacuolar structures designated as type II cytopathic vacuoles (CPV II). As shown in a previous communication from this laboratory, virus cores accumulate around these structures late in infection (9). Cores are also seen free in the cytoplasm (1, 9), and 140*S* structures which are biochemically and morphologically identical to cores are also found free in a cytoplasmic extract (2, 6, 7, 9, 13) The virion is formed by cores budding into the extracellular fluid through the plasma membrane (1, 9) or by the budding of cores into intracellular vacuoles (9). In either case, the outer membrane of the virion is acquired during the budding process.

The synthesis of protein and the synthesis of RNA directed by arboviruses appear to be related in that both processes take place on the viral replicative intermediate structure (4, 5). Because of this and because of the rapid association between newly formed protein and viral RNA, it was of interest to determine the effect of an inhibitor of virus protein synthesis on virus replication and assembly. Therefore, cells infected with Semliki Forest virus (SFV; arbovirus group A) were treated with cycloheximide during the log phase of virus replication. A rapid and marked inhibition of virus protein synthesis was observed with little or no effect on the rate of viral RNA synthesis; however, a marked increase in intracellular viral RNA was found after 1 hr of cycloheximide treatment. This accumulation of viral RNA appeared to result from an inability to form viral cores in the presence of cycloheximide. At the end of a 1-hr treatment with cycloheximide or other inhibitors of protein synthesis, CPV II did not form in the cytoplasm of treated cells, whereas they were readily identified in cells which had not been treated with inhibitor. These findings may reflect the lack of a large intracellular pool of the major protein of the SFV core.

MATERIALS AND METHODS

General methods. Virus pools and cells were prepared as previously described (8, 16). Viral RNA synthesis and protein synthesis were estimated by pulse

labeling of virus-infected and actinomycin D-treated chick embryo fibroblasts (CEF) late in infection. Under these conditions, more than 80% of the protein synthesis (4) and 95% of the RNA synthesis (16) being carried out are virus directed.

Sucrose density gradient analysis for RNA extracted with sodium dodecyl sulfate (SDS)-phenol was carried out by methods standard in this laboratory (4). The method of gradient analysis for 140S cytoplasmic viral cores has also been described in detail (7). Polyacrylamide gel electrophoresis of viral proteins and analysis of the results by autoradiography and microdensitometry were carried out by the methods of Summers et al. (15) and Fairbanks et al. (3), respectively.

Electron microscopy. Cultures were fixed with 3% glutaraldehyde and scraped from plastic dishes; the cells were sedimented. The pellets obtained were prepared for electron microscopy by established procedures (9). At least 25 cell profiles were examined in ultrathin sections of each sample.

RESULTS

Effect of cycloheximide addition on subsequent virus production. Cultures were infected with SFV at a virus to cell multiplicity of 20:1. At intervals after infection, cultures were harvested by rapid freezing, and 100 μg of cycloheximide per ml was added to duplicate cultures. All cycloheximide-treated cultures were harvested with the last culture frozen in the untreated series, that is 8 hr after infection. Virus titers were determined in cell cultures.

After addition of cycloheximide, no increase in virus titer was seen (Fig. 1). Similar results with Sindbis virus were recently reported (12). Since arboviruses are thermolabile, it is not clear from these studies whether virus synthesis stops completely or is greatly slowed after cycloheximide addition. Continued budding of virus was noted in electron micrographs for as long as 2 hr after cycloheximide addition (*see below*).

Effect of cycloheximide on virus protein and RNA synthesis. By 4 hr after SFV infection in CEF treated with actinomycin D, which inhibits cellular but not viral RNA synthesis, almost all of the protein and RNA synthesized are virus-directed (4, 16). Therefore, at this time, 100 μg of cycloheximide per ml was added to infected cultures, and 2-min pulse labeling of RNA or protein was performed with ^{3}H-uridine or ^{3}H-leucine, respectively, at 0, 30, or 60 min after the addition of cycloheximide. The results (Fig. 2) showed the expected, rapid and marked drop in the rate of viral protein synthesis but no significant alteration of the rate of viral RNA synthesis. Extending the time of exposure to cycloheximide to 2 hr also had no effect on viral RNA synthesis.

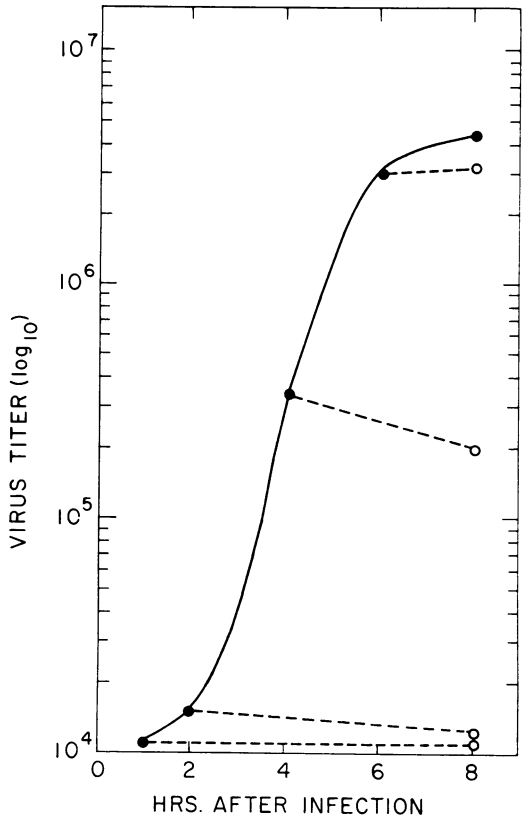

FIG. 1. *Virus growth in cycloheximide-treated cells. Chick cells were infected with SFV at a virus to cell multiplicity of 20:1. At the indicated times, cultures were rapidly frozen and cycloheximide (100 μg/ml) was added to identical cultures. All of the cycloheximide-treated cultures were frozen at the same time as the last culture taken, 8 hr after infection. All cultures were then titered for virus: ●, cultures taken for assay at indicated time; ○, cultures treated with cycloheximide at indicated time (dotted line) and allowed to incubate until 8 hr after infection.*

Nature of viral RNA formed in the presence of cycloheximide. The nature of the viral RNA formed in the presence of cycloheximide was investigated by labeling viral RNA for 1 hr with ^{3}H-uridine in actinomycin D-treated cells. This study was carried out 4 to 5 hr after viral infection in the presence or the absence of cycloheximide. All of the species of viral RNA present in control cells (Fig. 3A) were present in cycloheximide-treated cells (Fig. 3B). These RNA species included 42S and 26S single-stranded viral RNA and the ribonuclease-resistant viral replicative form and replicative intermediate (5, 7, 8, 11, 13).

Examination of Fig. 3 reveals one important and consistent difference between cycloheximide-

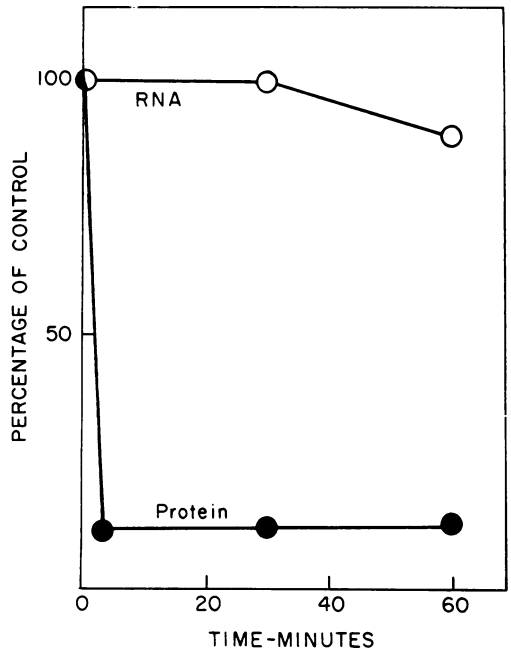

FIG. 2. *Protein and RNA synthesis in cycloheximide-treated cultures. Chick cells were treated with actinomycin D (1 μg/ml) and infected with SFV at a virus to cell multiplicity of 20:1. After 4 hr of infection, the cells were treated with cycloheximide for the indicated period of time. ^{3}H-uridine (20 μc/ml, 20 c/mM) or ^{3}H-leucine (20 μc/ml, 44 c/mM) was then added to the cultures for 2 min. The cultures were then washed, scraped in 0.1 M NaCl, and precipitated with 0.25 N perchloric acid. After washing, the precipitates were solubilized with 0.3 N NaOH. Acid-soluble counts were estimated in a scintillation spectrometer, and protein content was determined by the method of Lowry et al. (10). Results are reported as a percentage of specific activity of cultures not treated with cycloheximide.*

treated cells and controls. At least twice the level of counts present in the controls was found in the cycloheximide-treated group (note the change in scale). Since the rates of RNA synthesis were almost identical in the two groups (Fig. 2), this result suggests an intracellular accumulation of both forms of single-stranded viral RNA in the cycloheximide-treated group. The amount of ribonuclease-resistant RNA was about the same in both groups.

Cells were also exposed to cycloheximide for 1 hr before addition of ^{3}H-uridine. They were then incubated for an additional hour also in the presence of cycloheximide. (Total time in cycloheximide was 2 hr.) Under these conditions, somewhat different results were obtained (Fig. 4). Here, only the level of 26*S* RNA counts was markedly increased in the cycloheximide-treated group. In contrast to the results shown in Fig. 3, ribonuclease-resistant RNA and 42*S* RNA were labeled to about the same extent both in controls and in cycloheximide-treated cells in this experiment (Fig. 4).

It was of interest to determine the reason for the accumulation of viral RNA forms in the cytoplasm of cycloheximide-treated cells.

Failure to form viral cores in cycloheximide-treated cells. As previously shown, after 1 hr of labeling with RNA precursors in SFV-infected cells, the most heavily labeled cytoplasmic structure was the 140*S* viral RNA core (7). This structure was partially resistant to treatment with pancreatic ribonuclease at 1 C (Fig. 5A). In cycloheximide-treated cells, however, the counts were polydisperse and no 140*S* ribonuclease-resistant structure was present (Fig. 5B). Again, as noted previously, the level of total radioac-

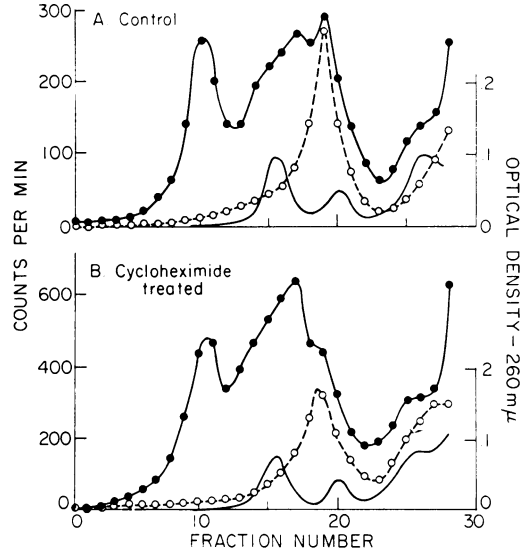

FIG. 3. *Viral RNA synthesis in cycloheximide-treated cells. Cells were infected and treated with actinomycin D as previously described. After 4 hr of infection, cycloheximide (100 μg/ml) and 20 μc/ml of ^{3}H-uridine were added to the cultures which were then incubated for an additional 1 hr. After 5 hr of infection, RNA was extracted from the cells and analyzed by sedimentation in sucrose density gradients (6 to 30% sucrose). Fractions of 0.15 ml were collected and 0.05-ml samples of these were analyzed for acid-precipitable radioactivity (●) and optical density at 260 nm (solid lines). Other 0.05-ml portions of the collected samples were treated with ribonuclease (2 μg/ml, 37 C, 30 min in 0.1 M NaCl) and were also analyzed for acid-precipitable radioactivity (○). A, control cultures; B, cultures treated with cycloheximide. The top of the gradient is to the right in this and subsequent figures.*

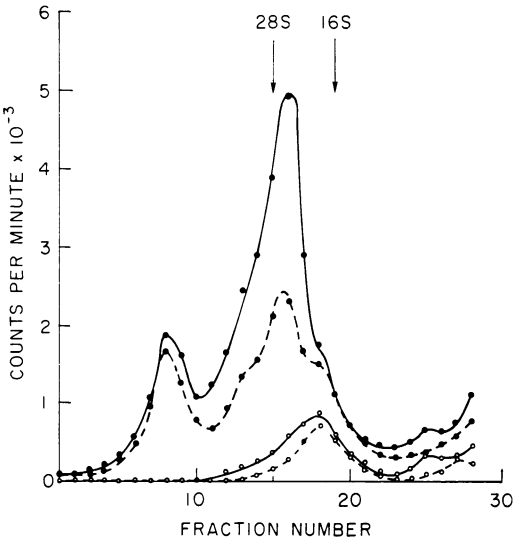

FIG. 4. *Viral RNA synthesis in cycloheximide-treated cultures. Cells were infected and actinomycin D-treated as previously described. After 4 hr of infection, cycloheximide (100 μg/ml) was added to half of the cultures. After 5 hr of infection, ^{3}H-uridine was added to cycloheximide-treated cultures and controls. Cycloheximide was not removed. After 6 hr of infection, RNA was extracted and analyzed as described in the legend to Fig. 3. Solid lines represent results of cycloheximide-treated cultures; dotted lines, control cultures; open circles, acid-precipitable radioactivity of ribonuclease-treated (2 μg/ml, 30 min, 37 C, 0.1 M NaCl) samples; closed circles, acid-precipitable radioactivity. The peaks of optical density at 260 nm are indicated by the designations 28S and 16S.*

tivity was much higher in the cycloheximide-treated group.

Cytoplasmic structures and virus budding in cycloheximide-treated cells. Late in the log phase of viral RNA replication (about 6 hr after infection), several CPV were easily identified in the cytoplasm of group A arbovirus-infected cells. These have been grouped into two general classes, CPV I and CPV II. CPV I are seen early in infection and together with the plasma membrane are sites of viral RNA synthesis. CPV II accumulate late in infection. They are membranous structures surrounded by 30-nm particles with ultrastructural characteristics of virus cores (9).

To determine the effect of cycloheximide treatment on the formation of these structures, cells infected 5 hr previously were treated for 1 hr with 100 μg of cycloheximide per ml and then rapidly fixed and prepared for examination in the electron microscope. Controls were taken of comparable, untreated cells after 5 or 6 hr of infection.

Typical CPV I were present at 5 hr after SFV infection and persisted after 1 hr of cycloheximide treatment (Fig. 6A). By 6 hr, CPV II could be easily found in controls (Fig. 6B). In cells treated with cycloheximide for 1 hr after 5 hr of infection, however, no CPV II were found after extensive examination of many fields.

Virus has been seen budding from the plasma membrane in electron micrographs of infected cells from very early in infection until severe cytoplasmic damage is present (1, 9). When cycloheximide was added for 1 or 2 hr after 5 or 6 hr of

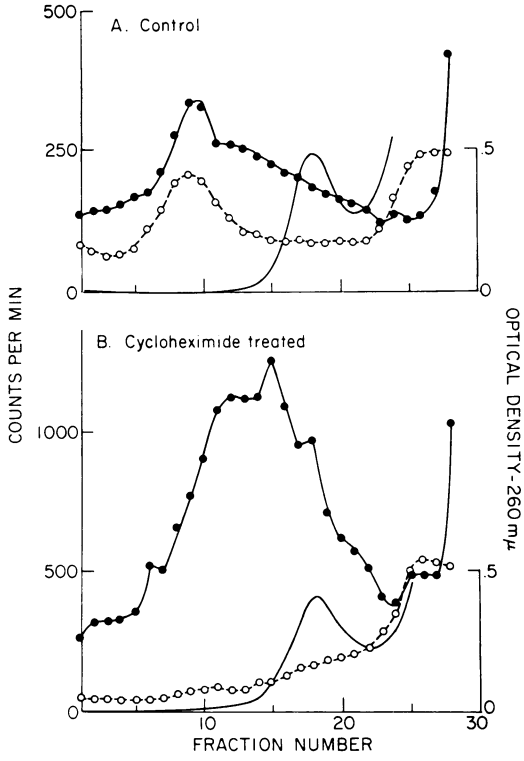

FIG. 5. *Failure to form cores in cycloheximide-treated cultures. Cultures were infected and actinomycin D-treated. After 4 hr of infection, 20 μc of ^{3}H-uridine per ml was added in the presence or absence of 100 μg of cycloheximide per ml. After 5 hr of infection, the cells were washed, scraped into RSB (0.01 M NaCl, 0.01 M Tris, pH 7.2, and 0.0015 M $MgCl_2$), and disrupted with a Dounce homogenizer. The nuclear fraction was removed by sedimentation, and half of each supernatant fluid was treated with ribonuclease (1 μg/ml, 1 C). The ribonuclease-treated samples and the balance of the extracts were layered over a 15 to 30% sucrose gradient in RSB and were sedimented for 1 hr at 100,000 × g in an SW 50 rotor. Samples were collected and assayed for acid-precipitable radioactivity and optical density at 260 nm (solid lines, peak represents 74S ribosomes). Symbols: ●, acid-precipitable radioactivity; ○, acid-precipitable radioactivity after ribonuclease treatment. A, untreated cell extract; B, extract from cycloheximide-treated cells.*

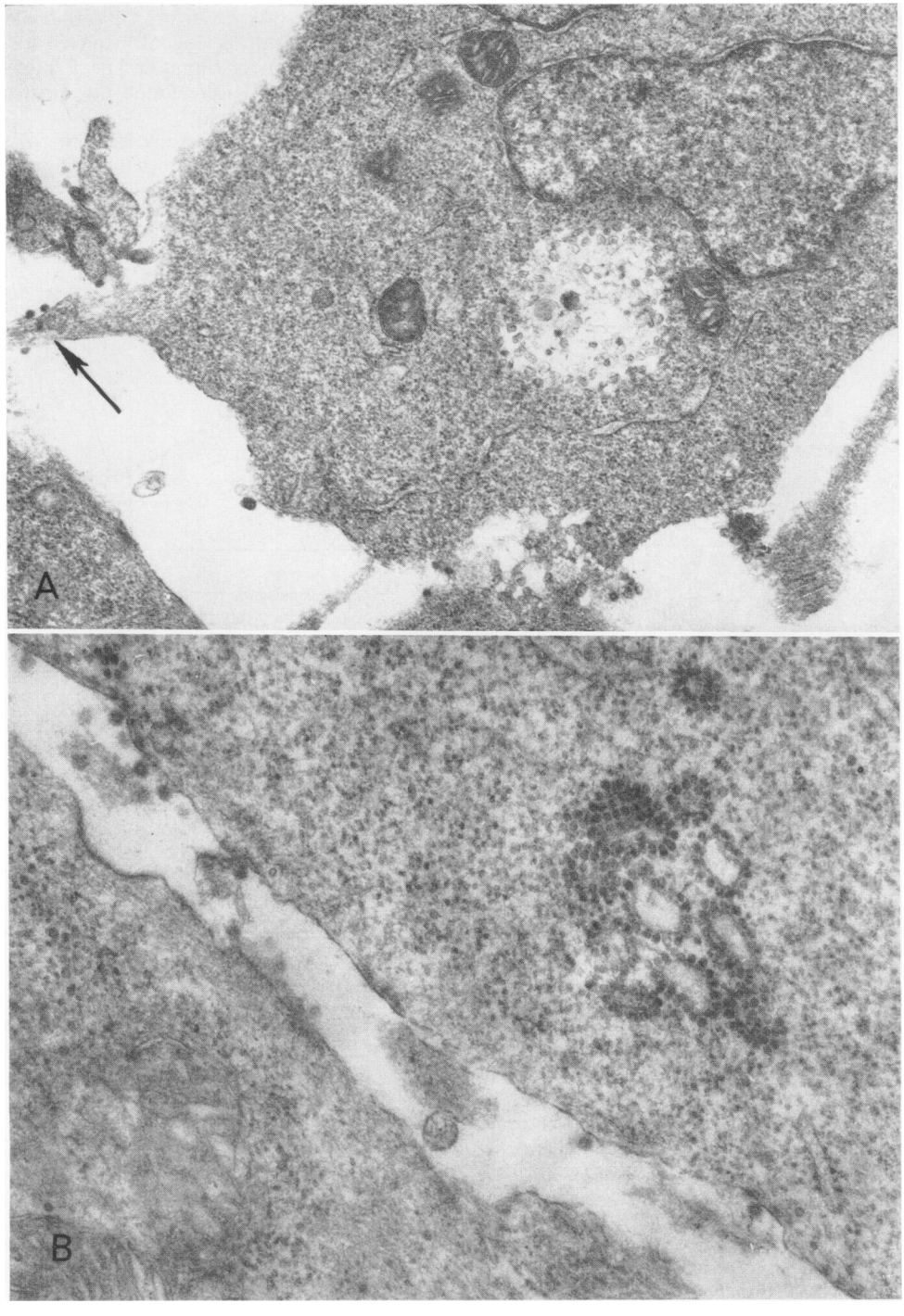

FIG. 6. *Cytoplasmic structures in SFV-infected cells. (A) CPV I in cell treated with cycloheximide for 1 hr after 5 hr of infection. Mature virus particles (arrow) continue to bud from the surface of a cell process. Uranyl acetate. × 24,000. (B) Typical cluster of CPV II in cell with abundant surface budding virus (6 hr after infection). Uranyl acetate. × 38,000.*

infection, budding virus cores were still found at the cell surface (Fig. 6A). This finding suggests that virion maturation may continue in the cells if cycloheximide is added at appropriate times. Addition of cycloheximide after only 3 hr of infection, however, completely suppressed virion formation within 2 hr (Table 1).

Because of the marked effect of cycloheximide on the formation of CPV II, additional experiments on this phenomenon were undertaken (Table 1). In experiment 1, cycloheximide was added at 2 or 3 hr after infection for an additional 2 hr. Examination of the cultures in this study (Table 1) showed surface-budding virus (SBV) first appearing at 4 hr after infection and CPV I appearing by 5 hr. In cultues to which cycloheximide had been added after 2 or 3 hr, SBV failed to appear. However, just as in controls, CPV I, which are RNA production sites (9), appeared at 5 hr after infection in cells treated with cycloheximide for 2 hr.

In experiment 2, cells were infected with a higher virus to cell multiplicity (40:1) than in the previously discussed experiments, so that both CPV I and II and SBV were present in controls by 5 hr after infection (Table 1). In this experiment, addition of cycloheximide, puromycin, or fluorophenylalanine for 1 hr also suppressed formation of CPV II; CPV I and SBV were unaffected.

Cytoplasmic location of the major core protein. The above results suggested that the cytoplasmic pool of the major core protein available for coating viral RNA may be very small, since inhibition of protein synthesis rapidly halts the production of viral cores. To determine whether a free cytoplasmic pool of this protein does exist, or whether the protein is sequestered in some manner, polyacrylamide gel electrophoresis was performed on several cytoplasmic fractions.

TABLE 1. *Electron microscopy of SFV-infected cells*

Expt	Duration of initial infection (hr)	Inhibitor[a]	Total duration of infection (hr)	CPV I	SBV	CPV II
1	2		2	−[b]	−	−
	3		3	−	−	−
	4		4	−	+	−
	2	Chx, 2 hr	4	−	−	−
	5		5	+	+	−
	3	Chx, 2 hr	5	+	−	−
2	5		5	+	+	+
	4	Chx, 1 hr	5	+	+	−
	4	FPA, 1 hr	5	+	+	−
	4	Puro, 1 hr	5	+	+	−

[a] Chx, cycloheximide (100 μg/ml); FPA, fluorophenylalanine (100 μg/ml); Puro, puromycin (50 μg/ml).

[b] Symbols: +, present; −, absent.

Actinomycin D-treated CEF were infected with a high multiplicity of SFV in the presence of guanidine which blocks SFV replication. After 4 hr, the cells were washed and virus infection was allowed to proceed. After 1 hr, almost all of the proteins synthesized under these conditions are produced under viral direction (6).

The proteins produced at this time were labeled with ^{14}C-amino acids, and a cytoplasmic extract was prepared. The extract was layered over a 15 to 30% sucrose gradient in reticulocyte buffer (RSB) and was sedimented for 1 hr. Fractions were collected, and the proteins of the 140*S* fraction, the top fractions, and the sediment were solubilized and fractionated by polyacrylamide gel electrophoresis (15). The gels were sliced, and the slices were dehydrated and exposed to X-ray film. The films were developed and analyzed in a microdensitometer (3).

The results of this experiment are summarized in Table 2. The significant finding was that, of all of the viral proteins identified, only the major core protein (protein 1) was not identified in the top component of the sucrose gradient. Since the latter component represents the pool of proteins not associated with a 140*S* structure and free in the cytoplasm, these results suggested that the free pool of this protein is very small in SFV-infected cells.

To quantitate this observation, the dehydrated gels were sliced into 1-mm strips after autoradiography. The strips were placed in scintillation vials with 1 ml of liquid scintillator and counted. The previously observed lines on autoradiography (Table 2) corresponded to incompletely resolved peaks in the electrophoretogram (Fig. 7). All of the polypeptides previously reported (6) were present in the extract of whole cytoplasm (Fig. 7C). The pellet also contained all of the peaks of radioactivity, but the distribution differed from that of the whole cytoplasm (Fig. 7A). In contrast, at most only a very small peak 1 (the major core protein) was found in the top component (Fig. 7B). Again, the distribution of radioactivity differed markedly from that of the whole cytoplasm. As previously reported (6; Table 2), the 140*S* fraction contained proteins 3 and 1, the former being only a minor component. T. Sreevalsan (*personal communication*) also observed a very low concentration of the core protein of another group A arbovirus, Sindbis virus, free in the cytoplasm.

TABLE 2. *Cytoplasmic location of viral proteins in SFV-infected cells*

Cytoplasmic fraction	Protein[a]				
	B	A	3	2 (Membrane protein)	1 (Major core protein)
Top component[b]	+	+	++	++	0
140S	0	0	+	0	++
Sediment	+	+	++	++	++
Whole cytoplasm	+	+	++	++	++
Virus[c]	0	0	+	++	++

[a] Symbols: 0, none present; +, a minor component present; ++, a major component present. Previous studies from this laboratory have indicated the presence of at least five proteins which are coded for by the virus. They are designated proteins A, B, 1, 2, and 3 (6).

[b] Chick cells (10^7) which had been treated for 1 hr with 2 μg of actinomycin D per ml were infected with SFV at a virus to cell multiplicity of 250 in the presence of 3 mg of guanidine per ml. After 4 hr, the cells were washed. After additional 1 hr, the cells were exposed to 10 μc of ^{14}C-amino acids per ml for 1 hr. A cytoplasmic extract was prepared, layered over a 15 to 30% sucrose gradient in 0.01 M NaCl, 0.01 M tris (hydroxymethyl) aminomethane (Tris; *p*H 7.2), and 0.0015 M $MgCl_2$ and sedimented for 1 hr at 100,000 $\times$ *g*. Fractions were collected, and the solubilized proteins of the top two fractions, the 140*S* fraction, and the sediment were analyzed by polyacrylamide gel electrophoresis (15). The gels were sliced and exposed to X-ray film (3). Microdensitometer tracings were then prepared from the developed films in a Joyce-Loebl microdensitometer.

[c] These results are taken from a previous publication from this laboratory (6).

DISCUSSION

These results indicate that in the presence of cycloheximide viral RNA is poorly, if at all, incorporated into its core particle or nucleocapsid. This result is consistent with earlier findings indicating that arbovirus core proteins are incorporated into cytoplasmic cores at a very rapid rate, almost as quickly as these proteins can be synthesized (2, 4). Apparently, only a small pool of the major core protein (protein 1) was present and almost none was free in the cytoplasm. The major core protein was therefore probably used avidly and its concentration might be one limiting factor in virus production.

As previously discussed (9), CPV II appeared to be associated with virus core formation. CPV II may develop in the cytoplasm as early as 4 hr after infection and are abundant after 6 to 8 hr (9). Several investigators have interpreted the 28-nm particles surrounding CPV II as virus cores or "precursor particles," and only one of several studies has suggested that the CPV II structure is not important in viral morphogenesis (9). Arbovirus protein synthesis is membrane-associated (2, 4), and transitions between the rough endoplasmic reticulum and CPV-II have been observed by us. These include membranous structures with both ribosomes and viral cores attached (P. M. Grimley and R. M. Friedman, J. Exp. Mol. Pathol., *in press*).

Our present observation that CPV II did not form in the presence of cycloheximide may indicate that the appearance of CPV II under normal conditions requires an excess of virus core formation (1). It is also conceivable that the normal appearance of CPV II reflects a marked decline in the rate of core release from membrane assembly sites. Present evidence does not distinguish between these possibilities. The persistence of virus budding for as long as 2 hr in the presence of

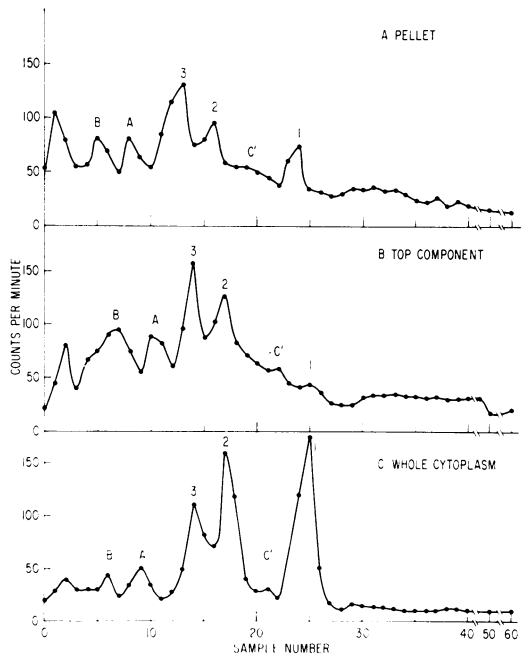

FIG. 7. *Electrophoretograms of polyacrylamide gels. The dehydrated gels prepared as described in the legend to Table 2 were, after autoradiography, sliced into 1-mm segments. The segments were placed in scintillation vials with liquid scintillator and counted. (A) Pelleted radioactivity; (B) top component; (C) whole cytoplasm. The peak marked C' has been previously noted in autoradiograms. It is uncertain whether it is a viral or host product (4). The other peaks are marked as indicated in Table 2.*

cycloheximide after 6 hr of initial infection indicates that a pool of cores must already have existed in the cytoplasm, since no new cores could be formed. This result is consistent with the biochemical findings of Sreevalsan and Allen in Western equine encephalitis infection (13). Reduction of virus budding when cycloheximide is added very early in infection (Table 1) suggests that the pool of available cores is significantly smaller before 4 hr.

In contrast to CPV II, CPV I formed quite well in the presence of cycloheximide, if the drug was added after RNA synthesis was well under way. CPV I persisted in the presence of cycloheximide. These results are consistent with the previously observed role of the CPV I in viral RNA synthesis (9) and with the failure of the drug to alter significantly the rate of viral RNA synthesis if added after RNA synthesis is initiated (12; Fig. 2). The latter finding incidentally indicates that the viral RNA polymerase must be stable for at least 2 hr.

The accumulation of viral RNA in the cytoplasm is also consistent with the above noted data. The rate of viral RNA synthesis was constant, but no RNA could leave the cells since assembly of cores was impaired. Interestingly, both 42*S* and 26*S* viral RNA accumulated but not the ribonuclease-resistant templates. The latter observation is consistent with the failure of cycloheximide to alter the rate of RNA synthesis. The accumution of both single-stranded forms of viral RNA (42*S* and 26*S*) may indicate that their formation is related since only 42*S* RNA, the form present in the virion, should be removed directly by virion release from the cell.

After 2 hr of cycloheximide treatment, only the 26*S* form of single-stranded RNA was increased in amount. A similar observation has been reported with Sindbis virus (12). Of the several possible explanations for this finding, we suggest that a viral protein is necessary for the conversion of 26*S* RNA to 42*S* RNA, and after 1 hr of cycloheximide treatment this protein is in short supply. We favor this notion for two reasons: (i) low doses of interferon inhibit the synthesis of 42*S* RNA to a much greater extent than that of 26*S* RNA (10) and (ii) studies with Sindbis virus temperature-sensitive mutants have suggested the same conclusion (C. M. Scheele and E. R. Pfefferkorn, Bacteriol. Proc., p. 196, 1969).

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