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Specific Membranous Structures Associated with the Replication of Group A Arboviruses[1]

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Intracytoplasmic membranous structures of a unique type were associated with the replication of three group A arboviruses: Semliki Forest virus (SFV), Sindbis virus, or Western equine encephalomyelitis virus. The structures, referred to as type 1 cytopathic vacuoles (CPV-1), were membrane-limited and characteristically lined by regular membranous spherules measuring 50 nm in diameter. The membranous spherules typically contained a fine central density, but were neither virus cores nor virions. Detection of CPV-1 by electron microscopy at 3 to 6 hr postinfection coincided with the time of rapid virus growth and preceded the accumulation of virus nucleocapsids. A range of 20 to 100 CPV-1 profiles were counted per 100 ultrathin cell sections at 6 to 9 hr postinfection when viruses were grown in chick embryo, baby hamster kidney, or mouse L cells. Maximum counts remained in the same range even when the multiplicity of infection was varied over 100-fold. Inhibition of cellular ribonucleic acid (RNA) and protein synthesis by actinomycin D during SFV infection did not decrease the counts of CPV-1; however, biogenesis of CPV-1 was decreased when viral replication was limited by inhibitors of viral RNA synthesis (guanidine) or of viral protein synthesis (cycloheximide). On the basis of present and earlier findings, we concluded that formation of CPV-1 must result from a virus-specified modification of pre-existing host cell macromolecules.

Host cell membranes evidently participate at several stages in the development of group A arboviruses. Envelopment of nucleocapsids, to form mature virions, occurs either at the cell surface membrane (1) or along cytoplasmic membranes (17), and accumulation of nucleocapsids around membranous vacuoles is characteristic (17, 22, 24, 26). Investigations of Semliki Forest virus (SFV) replication led to some initial observations that unique membranous structures could be found in the cytoplasm of chick cells as early as 3 hr after infection (11, 15). The structures consisted of membrane-limited vacuoles which enclosed regularly attached membranous spherules (1, 11, 15) and which remained intact after cell disruption (11). We referred to the integral structures as type 1 cytopathic vacuoles (CPV-1) (15), because they were the first evidence of subcellular alteration during SFV infection.

High resolution autoradiography of SFV-infected chick cells pulsed with ^{3}H-uridine showed that ^{3}H-labeled macromolecules were localized near CPV-1 (15). We interpreted this evidence to suggest that CPV-1 might be sites of arboviral replication (12, 15). Present investigations provide additional biological evidence that CPV-1 are arbovirus-specific structures and that they are associated with the replication of group A arboviruses under varied conditions of infection. In the accompanying paper, Friedman et al. present biochemical and ultrastructural evidence that CPV-1 could represent a membrane-associated replication complex similar to that demonstrated in picornavirus infections (6).

[1] Presented in part at the Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 4-9 May 1969.

MATERIALS AND METHODS

Viruses. SFV was the Kumba strain from an original stock of Alick Isaacs, Mill Hill, London, England. Pools were prepared in chicken embryo cells treated with actinomycin D (13). Later, a plaque-purified clone of this strain (RWGI) was obtained from Joseph Sonnabend, Mt. Sinai Hospital, New York, N.Y., and again was passaged in chicken embryo cells (20). Sindbis virus (Oie) was obtained from Samuel Baron, National Institute of Allergy and Infectious Diseases, Bethesda, Md. This virus originated in Malaya (1956) and came from mosquito pool MM 2215 (3-5-59) at Walter Reed Army Institute of Research, Washington, D.C. After passage in mouse brain two times and mouse embryo cells two times, it had been passaged in chicken embryo cells since 1961. The virus was passaged in primary mouse embryo

cells three times by H. Oie before use in our laboratory. Western equine encephalomyelitis (WEE) virus was obtained from Royce Lockhart at the Central Research Station, E.I. DuPont de Nemours Co., Wilmington, Del. This virus was isolated from a mosquito in the Rocky Mountains (Carl Eklund). It was originally passaged in mouse brain 100 times, but has been carried in chicken embryo cells since 1955. A medium-size plaque clone was selected in 1962.

Cell cultures. Chicken embryo cells were prepared as primary monolayer cultures by previously described techniques (13). Baby hamster kidney cells (BHK) were originally a gift from Joseph Sonnabend and were propagated in suspension cultures. Mouse L cells (L-929) were obtained from Charles Buckler, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Method of infection. Virus was adsorbed in the presence of actinomycin D (1 μg/ml) at either 4 or 37 C for 1 hr. In the former method, cells were maintained at 4 C for up to 16 hr after adsorption to ensure a synchronous infection. Differences in the absorption technique proved to have no effect on observed morphological or biochemical events, and in most experiments the absorption was carried out at 37 C. Time of infection was considered to begin with the addition of virus at 37 C or when cultures were rewarmed to 37 C. Virus titers were determined by plaque assay in chicken embryo monolayers as described previously (35).

Reagents. Actinomycin D was a gift from Merck, Sharpe and Dohme Research Laboratories (Rahway, N.J.). Guanidine was purchased from Eastman Organic Chemicals (Rochester, N.Y.). Cycloheximide was purchased from Calbiochem (Los Angeles, Calif.).

Electron microscopy and cytochemistry. Infected cell monolayers were detached with a rubber policeman, sedimented for 10 min at 780 $\times$ *g*, and fixed with 3% glutaraldehyde in 0.1 M phosphate buffer at *p*H 7.2. Cell suspensions were similarly sedimented prior to fixation of the pellet. Subsequent steps in preparation for electron microscopy have been detailed previously (15). The acid phosphatase method was a modification of the Gomori technique (8). Samples were fixed in 2% glutaraldehyde in cacodylate buffer (*p*H 7.2) with 7% sucrose and incubated in the reaction mixture at 37 C for up to 2 hr. Ultrathin sections embedded in epoxy resin were stained with uranyl acetate or lead citrate before examination in an RCA EMU3F at 50 kv or an Hitachi 11E at 75 kv.

Counts of CPV-1 were performed in a predetermined number of unobscured ultrathin cell sections (ca. 800 nm) without regard to the plane of section or area of cell included.

Isolation of SFV ribonucleic acid (RNA) polymerase. At 5.5 hr postinfection, the cells were harvested as described in the accompanying paper. Cell pellets were suspended in 5 ml of hypotonic buffer containing 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.4), 1 mM $MgCl_2$, and 10 mM β-mercaptoethanol for 15 min at 4 C. Cells were disrupted (15 strokes) in a Dounce homogenizer. After centrifugation for 10 min at 800 $\times$ *g*, the supernatant cytoplasmic extract was centrifuged for 15 min at 13,000 $\times$ *g* in a Sorvall SS34 rotor. The resulting pellets were each resuspended in 5 ml of hypotonic buffer and resedimented at 800 $\times$ *g* for 5 min. The supernatant fluids were centrifuged for 15 min at 13,000 $\times$ *g*, and the pellets were resuspended in a small volume of hypotonic buffer (ca. 0.6 ml).

RESULTS

Ultrastructure and cytochemistry. The ultrastructure of CPV-1 in SFV-infected chicken cells has already been described (15). Identical structures were now found in cells infected by Sindbis or WEE virus. As illustrated in Fig. 1, membranous spherules measuring 50 nm in diameter were regularly attached to the lining of vacuoles in the cytoplasm. The vacuoles were membrane-limited and varied in cross-sectional diameter (Fig. 1). This was probably due to both actual differences in size and the different planes of random ultrathin sections. The average diameter of CPV-1 was 1 to 2 μm, but vacuoles of up to 5 μm were observed rarely. The spherules were always formed of a trilaminar "unit" membrane, and at high magnification a threadlike central density could usually be demonstrated (Fig. 2). Thus the spherules were larger than the nucleocapsids of group A arboviruses (ca. 28 nm) and also lacked the true core found in mature virions. The CPV-1 appeared ultrastructurally identical in different cell types (Fig. 1). As shown previously (15), the 50-nm spherules could sometimes be observed in patches along the cell surface (Fig. 3). This phenomenon may possibly be explained by fusions of CPV-1 with the cell surface (exocytosis) and consequent eversion of the spherule-lined vacuoles. Experiments with thorotrast (15) have already demonstrated that CPV-1 do not form by invagination of surface membrane. The cytochemical reaction for acid phosphatase showed enzyme localization in cisternae and vesicles of the Golgi apparatus of uninfected chicken cells and in both the Golgi apparatus and CPV-1 of SFV-infected chicken cells (Fig. 4).

Comparison of viruses in chicken cells. SFV, Sindbis or WEE viruses were serially passaged in chicken embryo cells, and typical growth curves obtained in our laboratory are illustrated in Fig. 5. The shape of the growth curves was similar, with an exponential phase occurring within 3 to 7 hr after infection. In parallel experiments, we examined the ultrastructural developments. To standardize morphological comparisons, at least 100 random sections of the chicken cells were surveyed at 1- or 2-hr intervals after infection with each of the viruses. In repeated infections with SFV or Sindbis viruses, CPV-1 appeared at 3 to 4 hr, and surface budding of virions could be detected at the same times. The growth of WEE in chicken embryo cells was less efficient than the

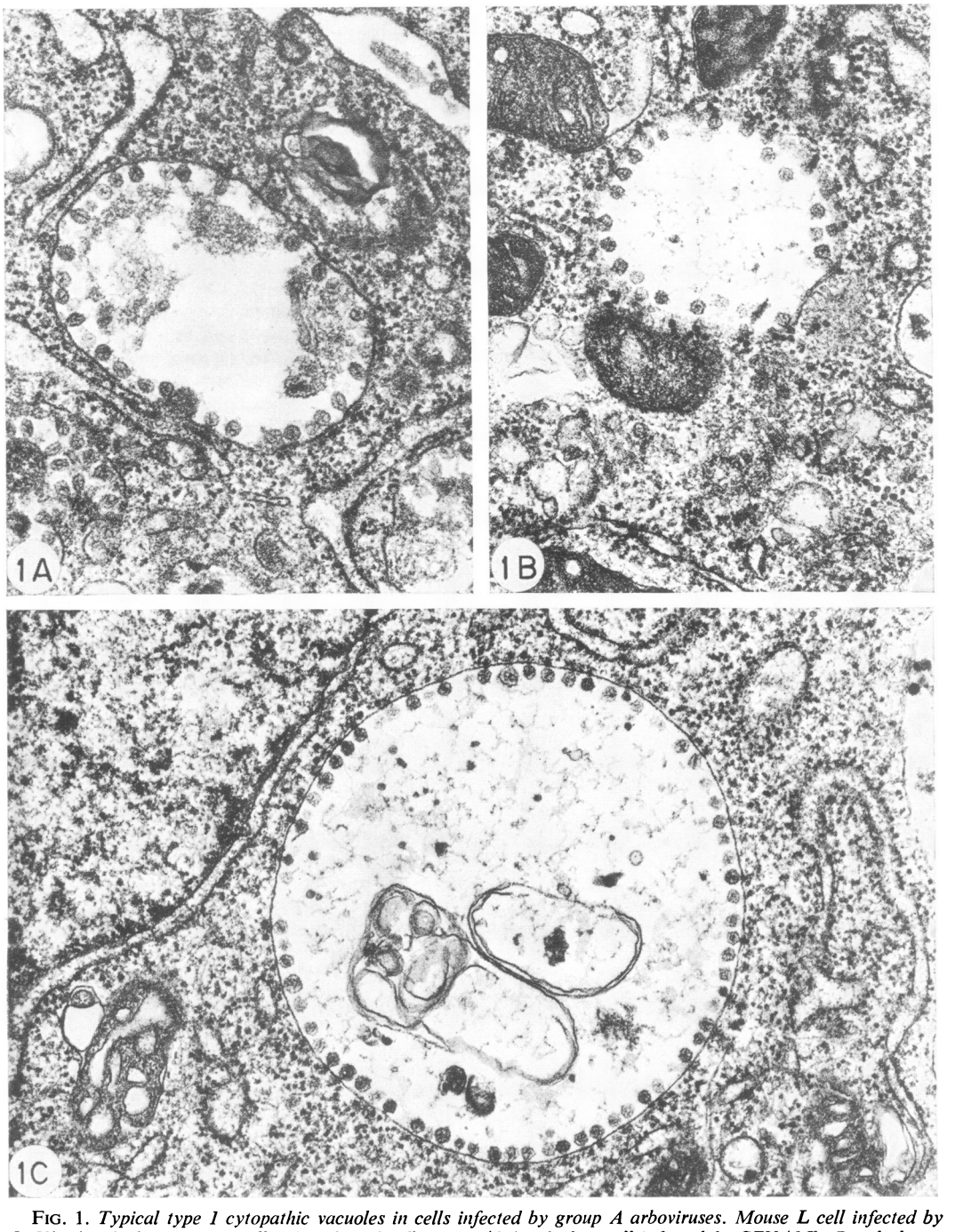

FIG. 1. *Typical type 1 cytopathic vacuoles in cells infected by group A arboviruses. Mouse L cell infected by SFV (1A); chicken embryo cell infected by Sindbis virus (1B); chicken cell infected by SFV (1C). Regularly arranged membranous spherules (50 nm in diameter) lining the interior surface of the vacuoles are a characteristic feature. The vacuoles vary in size, and large ones may contain membranous debris (1C).* ×*32,000.*

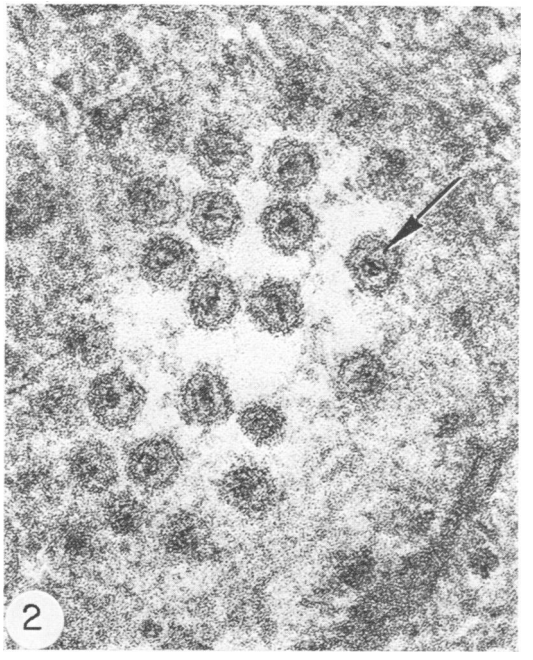

FIG. 2. *High magnification of spherules in chicken embryo cell infected by Semliki Forest virus. Note unit membrane (arrow). Threadlike density within the spherules is characteristic.* ×*130,000.*

growth of either SFV or Sindbis viruses (Fig. 5), and CPV-1 were not observed until at least 6 hr after WEE infection. Surface budding of virions also was not detected until this later time.

Because CPV-1 were well defined structures, although of varied sizes, we considered that counts in random samples of 100 cell sections might provide a useful index of the morphological developments with different viruses. In repeated experiments, counts of CPV-1 ranged from 10 to 20 per 100 cell sections at 3 to 4 hr after infection with SFV or Sindbis virus. By 6 to 8 hr postinfection, the typical range of counts was 20 to 60 CPV-1 per 100 cell sections, with a maximum of 99 CPV-1 found in one of the many experiments with SFV. During infections with WEE, the counts ranged from 10 to 20 CPV-1 at 8 to 12 hr. These lower counts with WEE appeared to correlate with the lower yield of infectious virus (Fig. 5). Considering geometric limitations of the thin-section sampling method (37), small differences in the individual hourly counts of CPV-1 during SFV and Sindbis infection did not appear significant; nevertheless, there was a repeated trend of a several-fold increase in counts between 6 to 8 hr postinfection as compared with 3 to 4 hr postinfection. The counts at 12 hr or later were never higher, and usually lower, than the counts at 6 to 8 hr.

Accumulation of virus nucleoids after 8 hr of infection is a characteristic feature of group A arbovirus infections (1, 12, 22, 26) and again was found in the present experiments. We previously referred to accumulations of SFV nucleocapsids around membrane-limited vacuoles as type 2 cytopathic vacuoles (CPV-2) because they occurred consistently later than CPV-1 (12, 15). This observation was confirmed in the present experiments with SFV, Sindbis, and WEE. CPV-2 were rarely observed before 6 hr after infection, and maximum numbers (up to 80 per 100 cell sections) were found after 12 hr.

CPV-1 in mammalian cells. The experiments in chicken embryo cells showed that the formation of abundant CPV-1 was not dependent upon infection with a specific group A arbovirus. We now wished to learn if formation of CPV-1 might be dependent upon the host cell type. Although the yield of SFV in BHK or mouse L cells (Fig. 6) was generally lower than that obtained in chicken embryo cells (Fig. 5), the kinetics of virus growth appeared to be similar. As in the chicken cells, CPV-1 were observed at 3 to 4 hr after infection and during the phase of exponential virus replication; however, the numbers of CPV-1 at early times appeared to be slightly smaller (2 to 10 per 100 cell sections). Maximum counts of CPV-1 in the BHK or L cells infected by SFV occurred at 6 to 12 hr after infection and were then in a range comparable (20 to 50 per 100 cell sections) to the counts found in chicken cells. Attention was focused upon the growth of SFV in BHK and L cells because the cell lines were vigorous and might prove useful for future biochemical comparisons; however, CPV-1 were also numerous when growth of SFV or WEE was tested in primate cells (*unpublished data*).

Multiplicity of infection and virus pools. The "virus-like" size and morphology of the membranous spherules within CPV-1 (Fig. 2) led us to question whether they might represent incomplete or defective virus particles. It is known, for example, that defective interfering particles can accumulate after repeated passages of an arthropod-borne rhabdovirus (vesicular stomatitis) at high multiplicities of infection in certain cell types (18). When virus pools contain such defective virus, yields of infectious virus can be improved by decreasing the multiplicity of infection. Fig. 7A illustrates that the maximum titers of SFV after a low multiplicity of infection (0.1 PFU per cell) were, in fact, slightly lower than after a high multiplicity of infection (10 PFU per cell). The kinetics of virus growth was altered only by a shift of the logarithmic phase to a

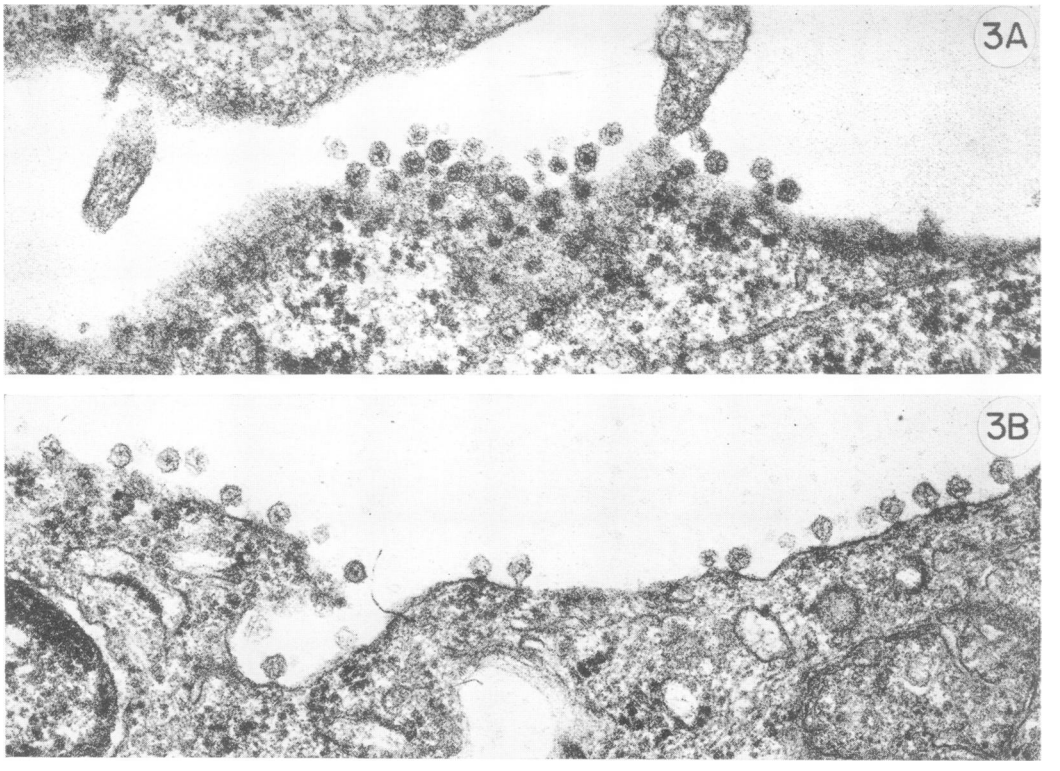

FIG. 3. *Membranous spherules at surface of cells infected by Semliki Forest virus. Surface of chicken embryo cell (A); surface of baby hamster kidney cell (B). Note arrangement of spherules in small patches, suggesting origin by fusion of type 1 cytopathic vacuoles (Fig. 1) with cell surface.* ×*48,000.*

later time. Differences in titer during the early hours of infection (Fig. 7A) were evidently narrowed by repeated cycles of infection. The counts of CPV-1 (Fig. 7B) paralleled the early differences in virus growth and became similar as infection progressed. In additional experiments, the multiplicity of infection was varied up to 500-fold with similar results. Comparisons of counts of CPV-1 at 6 hr after infection, when maximal numbers may be found, showed no more than 10-fold differences (Fig. 8). In some of these experiments, the inoculum virus was obtained from pools grown in mouse brain (17) rather than chicken embryo cells. Again, there was no remarkable difference in virus titers or counts of CPV-1.

Because CPV-1 have been associated with viral RNA synthesis by autoradiography (15) and cell fractionation experiments (accompanying paper), we also examined viral RNA polymerase activity and viral RNA synthesis in the high- and low-multiplicity infections. Assay of viral polymerase activity also showed minimal differences during the logarithmic growth phase. At 5.5 hr after infection, when the virus titer differed by about 10-fold, polymerase activity differed by about 3-fold (Fig. 9). In other experiments, viral RNA was extracted from cells infected with SFV at high and low multiplicities of infection following a 1-hr incubation with ^{3}H-adenosine and ^{3}H-uridine between 5 and 6 hr postinfection. The specific radioactivity of total viral RNA was not significantly affected by variations in multiplicity of infection (*unpublished data*). Furthermore, analysis of the viral RNA samples by polyacrylamide gel electrophoresis revealed all of the RNA forms previously identified in SFV-infected chick cells (20), and there were no striking differences in the proportions of the replicative intermediate, replicative forms, or 42*S* or 26*S* RNA species. These findings were thus consistent with other evidence that the presence of CPV-1 was related to active virus replication at various multiplicities of infection.

Treatment of cells with actinomycin D. The correlations of virus growth and counts of CPV-1 observed by electron microscopy suggested that biogenesis of CPV-1 was closely related to the

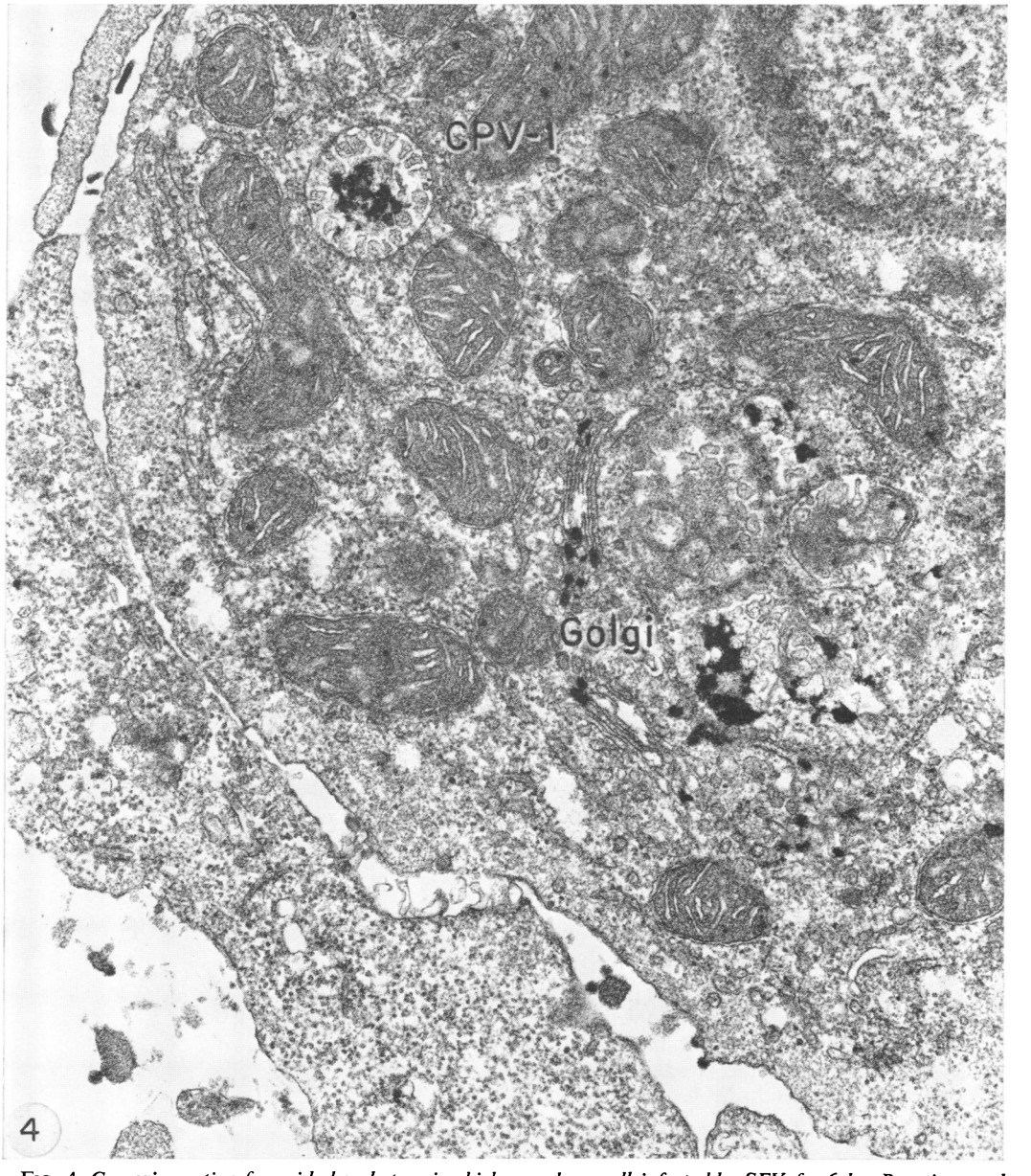

FIG. 4. *Gomori reaction for acid phosphatase in chicken embryo cell infected by SFV for 6 hr. Reaction product is localized within type 1 cytopathic vacuoles (CPV-1) and in cisternae or vesicles of the Golgi region.* ×*32,500.*

formation of infectious virus, irrespective of the host cell. We then questioned whether host cell synthetic functions were required. This was partially answered by evidence that the numbers of CPV-1 were similar in cells infected by SFV and Sindbis virus, even though SFV depresses host cell transcription (9, 35) more rapidly and effectively than Sindbis (3, 36). Addition of actinomycin D supported the evidence that biogenesis of abundant CPV-1 continues in the absence of significant host cell transcription. Actinomycin D was added at levels (up to 2 μg/ml) which effectively block ^{3}H-uridine incorporation by uninfected chicken embryo cells (3, 21, 35) and which inhibit amino acid incorporation by more than 80% within 4 hr (9). The yield of SFV, as shown by Taylor (35), was increased at least 50% in the cells pretreated with actinomycin D

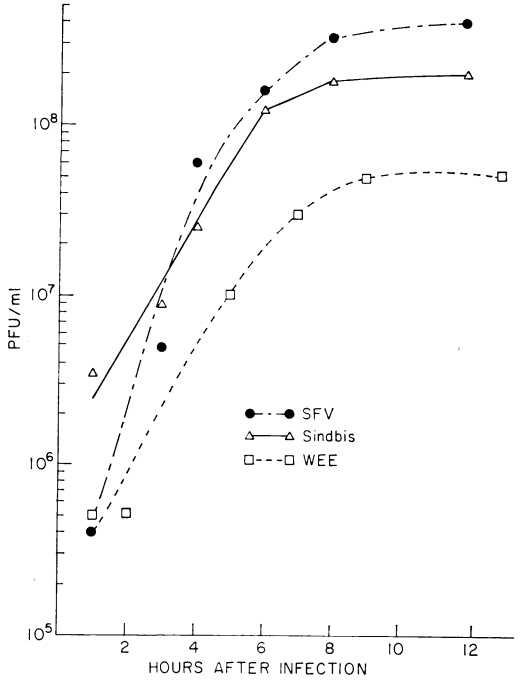

FIG. 5. *Growth of group A arboviruses in chicken embryo cells. Virus at a multiplicity of 20 PFU per cell was adsorbed for 1 hr at 37 C in tubes with 1 × 10^6 cells in serum-free Eagle medium containing 1 μg of actinomycin D per ml. After washing the cells three times in Eagle medium, incubation was continued in the presence of 0.1 μg of actinomycin D per ml. Points represent the yield from duplicate samples assayed for plaque formation on chicken cell monolayers (35).*

(Fig. 10). In the same experiment, there was no appreciable decrease in the overall counts of CPV-1 up to 15.5 hr, although their development may have been slightly delayed (Table 1).

Inhibition of viral replication. In view of the above correlations, we anticipated that biogenesis of CPV-1 would depend upon continued expression of viral functions. This presumption was supported by experiments in which virus replication was inhibited by cycloheximide or guanidine. SFV protein synthesis is almost totally blocked within a few minutes after the addition of cycloheximide (12), whereas guanidine inhibits the viral RNA synthesis up to 90% within 10 min (10). The experiments were designed so that numbers of CPV-1 could be compared at 6 hr after infection when their development would normally be maximal. Initial counts of CPV-1 per 100 chicken cell sections were made at the time when inhibitor was added. In the same experiment, parallel samples were treated with the inhibitor for specified intervals up to 6 hr postinfection and counted at the latter time. Infected cells, not treated with inhibitor, served as the controls and also were counted at the end of the experiment. As shown in Table 2, cycloheximide added before 2 hr of infection completely prevented the biogenesis of CPV-1; however, addition of cycloheximide at 2 or 3 hr postinfection did not prevent the appearance of a few CPV-1. Addition of cycloheximide at 4 hr after infection apparently had no significant effect. In the experiments with guanidine (Table 3), there was evidently an inhibition of CPV-1 when the

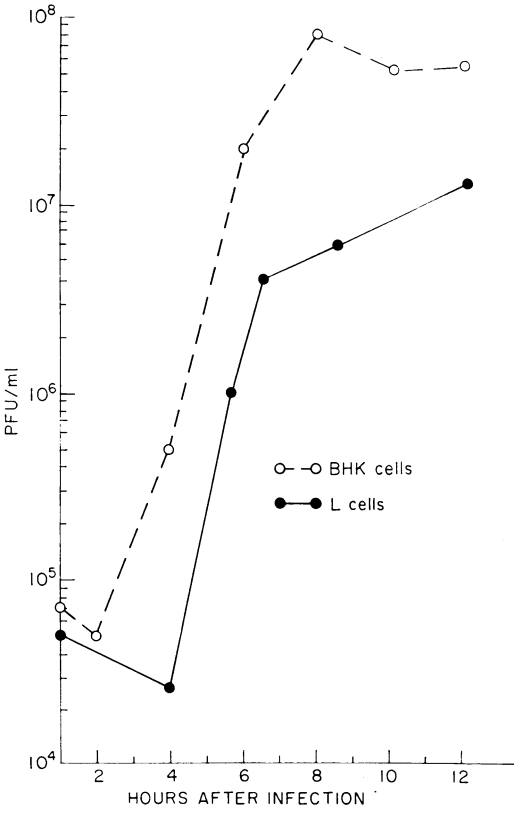

FIG. 6. *Growth of Semliki Forest virus (SFV) in baby hamster kidney (BHK) cell suspensions and in L cell monolayers. Method for L cells was as in Fig. 5, except that cells were grown on plates (1 × 10^7 cells). BHK cells (1 × 10^8 cells) were adsorbed with SFV at a virus to cell ratio of 10 in a volume of 5 ml. After 15 hr at 4 C, the cells were warmed to 37 C and incubated for 30 min in the presence of actinomycin D (0.5 μg/ml). The cells were then diluted to 200 ml with Eagle Spinner medium containing 2.5% fetal bovine serum, and incubation was continued at 37 C. At the indicated times, portions of the culture were removed and subsequently assayed for plaque formation on chicken cell monolayers (13, 35). Zero time was considered to be the time at which the Eagle Spinner medium was added.*

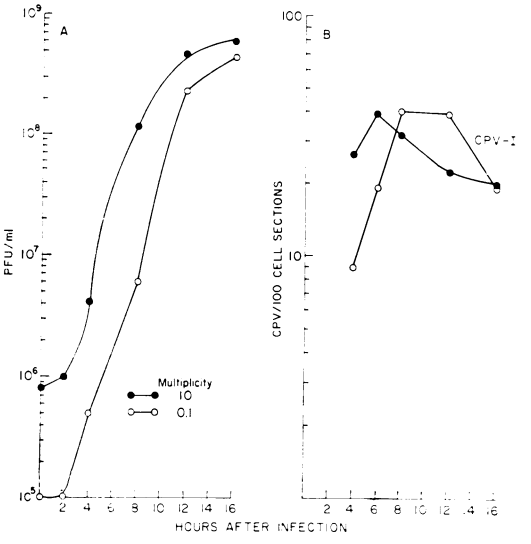

FIG. 7. *A, Growth of Semliki Forest virus (SFV) in chicken embryo cells at different multiplicities of infection. Method was as in Fig. 5. B, Counts of viral structures in a parallel set of chicken cells cultures on plates (2 × 10⁷). and simultaneously infected with the appropriate dilutions of SFV. Type 1 cytopathic vacuoles (CPV-1) were counted in 100 random, thin sections of chicken cells in each experiment.*

drug was added at 2 hr postinfection, and a slight reduction in the counts when the drug was added at later times.

The maximal effect of cycloheximide at early times of SFV infection (Table 2) could have been related to a secondary effect on RNA replication as shown previously with Sindbis virus (29). Comparison of viral RNA samples from control and cycloheximide-treated chicken cells showed that addition of the drug at 2 or 4 hr after infection with SFV had little effect on total RNA synthesis measured at 3 hr (Fig. 11) or 5 hr (12), respectively. When, however, the RNA made between 2 to 3 hr after infection was analyzed by polyacrylamide gel electrophoresis (20) an effect of cycloheximide treatment on the distribution of viral RNA forms was apparent (Fig. 11). Most striking, was the relative reduction in amounts of replicative intermediate and replicative forms. Presumably, this effect of cycloheximide was consequent to a block in the synthesis of viral polymerase protein rather than to any new type of action.

DISCUSSION

The membranous structures described as CPV-1 are morphologically unique. Other vacuoles of a multivesicular type have been described in virus infections (4, 5, 31), but only CPV-1 are differentiated by the regular attachment of 50-nm membranous spherules with a fine central density. The nature of the spherules remains unexplained, and an obvious consideration would be that they represent an incomplete form of virus consisting of membranous envelopes without the complete nucleic acid core. Although such incomplete particles have been described in cells infected by Sindbis virus (25) and influenza virus (23), present findings do not support the hypothesis that the membranous spherules are incomplete arbovirus nor that CPV-1 are an expression of infection by defective arbovirus or a "satellite" virus. Not only did CPV-1 form in a variety of cells infected by any of three group A arboviruses, but passage of SFV through different cells or inoculation of SFV or Sindbis at different multiplicites of infection had minimal effect on the numbers of CPV-1 counted at 6 hr after infection.

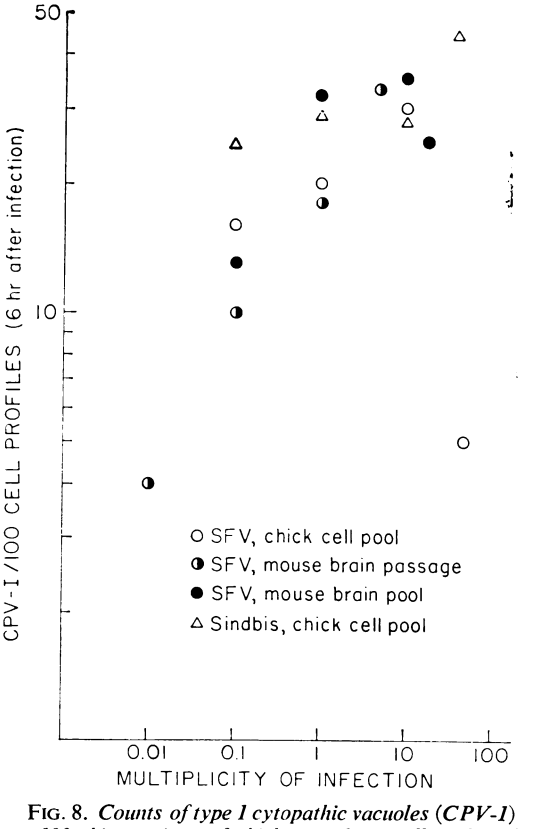

FIG. 8. *Counts of type 1 cytopathic vacuoles (CPV-1) per 100 thin sections of chicken embyro cells infected with some different multiplicities of Semliki Forest virus (SFV) or Sindbis virus. Method was as in Fig. 7B. In the experiments with SFV, the inoculum was obtained either from chicken cell or mouse brain pools.*

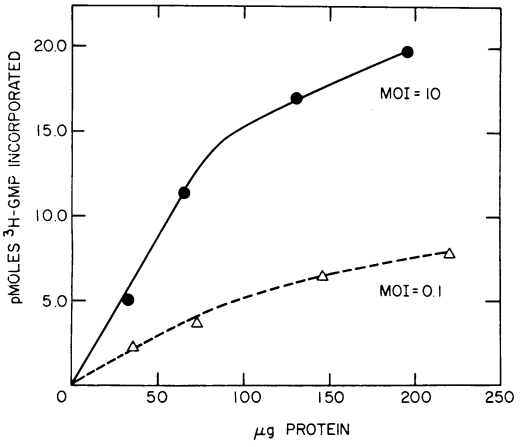

FIG. 9. *The effect of multiplicity of infection on Semliki Forest virus (SFV) RNA polymerase activity. Chicken embryo cell monolayers were adsorbed at 4 C with SFV at a multiplicity of 10 or 10^{-1}. After 15 hr, fresh Eagle medium was added, and the cells were warmed to 37 C. At 5.5 hr postinfection, the cells were processed according to the procedures given in Materials and Methods. Polymerase activity was assayed as a function of protein concentration in 0.1-ml reactions, as described in the accompanying paper, except that the final concentration of actinomycin D in the reaction was 1 μg/ml and the incubation was carried out at 28 C for 15 min. Multiplicity of infection (MOI) = 10 (●); MOI = 0.1 (△).*

The possibility of a passenger virus in SFV pools had been excluded previously (20). Furthermore, CPV-1 have been identified in suckling mouse brain infected by SFV (17) or Eastern equine encephalomyelitis virus (24) and during single cycle infections of mouse striated muscle (16). Although CPV-1 have never been observed in other than group A arbovirus infections, similar membranous vesicles have been observed in the Golgi region of cells infected with spring-summer encephalitis virus (19) and yellow fever virus (Grimley and Young, *unpublished data*). If these vesicles are homologous to the membranous spherules in CPV-1, they may constitute a morphogenetic link in the development of group A and group B arboviruses.

The experiments with actinomycin D-pretreated cells showed that formation of CPV-1 did not require transcription of cellular RNA and that participation of cellular proteins formed after 4 hr of infection must be minimal, if at all significant in their biogenesis. At a high multiplicity of infection (10 PFU per cell), SFV itself depresses the rate of total chicken cell protein synthesis by almost 90% in 4 hr, whereas the rate of cell-directed protein synthesis in SFV-infected chicken cells pretreated with actinomycin D is less than 2% of uninfected controls (9). Surprisingly, the demand for membranes during arbovirus envelopment and the biogenesis of large numbers of CPV-1 also does not appear to be accompanied by a specific stimulation of host cell lipid metabolism in Sindbis infection (28, 36), and some preliminary experiments by Friedman (*unpublished data*) failed to suggest any differential incorporation of ^{3}H-choline by the membrane fraction 5 density = 1.16 g/cm^3) of SFV-infected chicken cells, which include CPV-1 (see accompanying paper). These findings with arboviruses contrast with results reported for

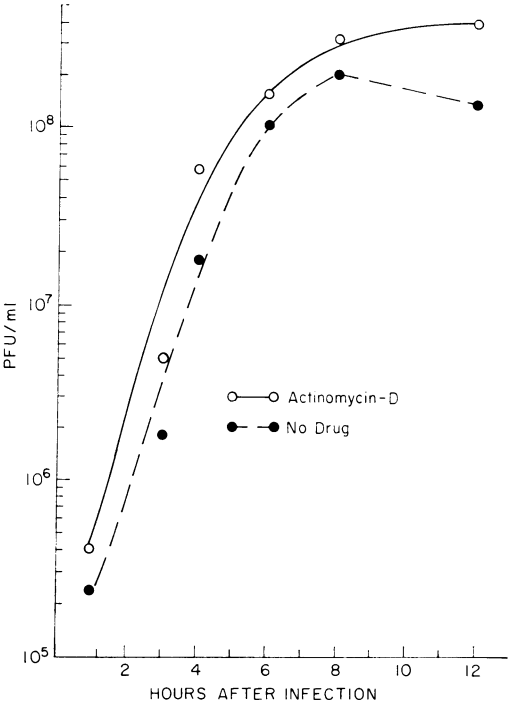

FIG. 10. *Growth curve of Semliki Forest virus (SFV) in chicken embryo cells with (○) or without (●) actinomycin D. SFV at a multiplicity of 45 PFU per cells was adsorbed at 37 C in tubes with 1.4 × 10^6 cells. Remainder of method was as in Fig. 5.*

TABLE 1. *Effect of actinomycin D (ACD) on counts of type 1 cytopathic vacuoles (CPV-1) in chicken embryo cells*[a]

Treatment	CPV-1 per 100 cell sections after infection with SFV (hr)[b]				
	3	5	7	9.5	15.5
Control	11	20	32	45	29
ACD	N[b]	18	20	35	22

[a] Same experiment as Fig. 10, except cell monolayers were grown in plates (2 × 10^7 cells).

[b] SFV, Semliki Forest virus; N, None observed.

TABLE 2. *Effect of cycloheximide on counts of type 1 cytopathic vacuoles (CPV-1)*

Cycloheximide added[b]		Counts of CPV-1 per 100 cell sections (Time after infection)[a]	
		5 hr	6 hr
No. 1	2 Hr	0	
	3 Hr	5	
	Controls[c]	25	
No. 2	1 Hr		0
	2 Hr		0
	3 Hr		6
	Controls[c]		32
No. 3	2 Hr		7
	4 Hr		42
	Controls[d]		58

[a] In different experiments, chicken embryo cells were infected with Semliki Forest virus at a multiplicity of 20 PFU/cell (1), 10 PFU/cell (3), or 0.1 PFU/cell (2).

[b] Cycloheximide (100 μg/ml) was added at specified times after infection.

[c] Initial counts showed that no CPV-1 were present when cycloheximide was added at 1 to 3 hr.

[d] Initial counts showed no CPV-1 at 2 hr and 49 CPV-1 at 4 hr.

TABLE 3. *Effect of guanidine on counts of type 1 cytopathic vacuoles (CPV-1)*

Time guanidine was added	Counts of CPV-1 per 100 cell sections 6 hr after infection[a]
2 Hr	8
3 Hr	13
4 Hr	15
Controls[b]	21

[a] Chicken embryo cells were infected with Semliki Forest virus (SFV) at a multiplicity of 10 PFU/cell. Guanidine (10 mM) was added at 2 to 4 hr after infection.

[b] Initial counts showed no CPV-1 at 2 hr, 4 CPV-1 at 3 hr, and 6 CPV-1 at 4 hr.

picornavirus infections, where lipid metabolism is stimulated and cytomembranes proliferate (2, 27), and lead us to conclude that biogenesis of CPV-1 must result from a virus-induced modification of pre-existing host cell macromolecules. A common localization of acid phosphatase enzyme in the Golgi apparatus and the CPV-1 of SFV-infected chicken cells suggested that membranes of the Golgi cisternae or the small Golgi vesicles could have been utilized by arboviruses during the formation of CPV-1.

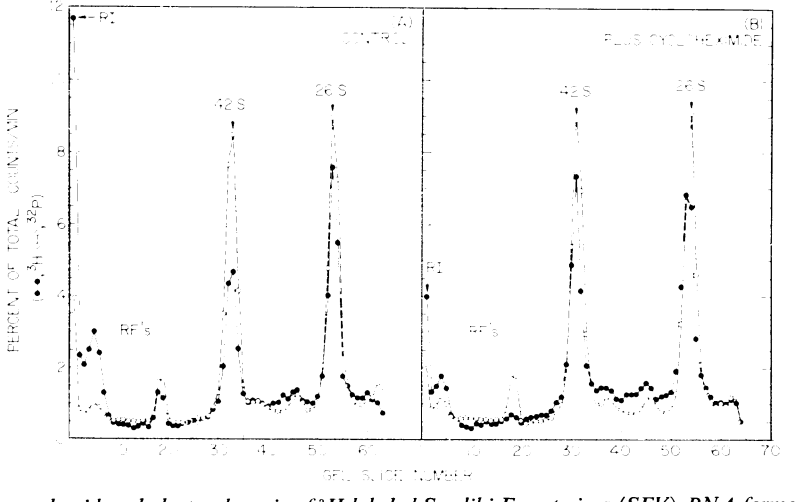

FIG. 11. *Polyacrylamide gel electrophoresis of ^{3}H-labeled Semliki Forest virus (SFV) RNA formed in the presence or absence of cycloheximide. Monolayers of chicken embryo fibroblasts were infected with SFV at a multiplicity of 10^{-1}. After 1 hr at 4 C, Gey medium containing 10% fetal bovine serum and 1 μg of actinomycin D/ml was added, and the cells were maintained at 4 C for 15 hr. The cells were rewarmed to 37 C. After 2 hr the medium was replaced with Eagle medium containing ^{3}H-adenosine and ^{3}H-uridine (100 μCi/ml each). Half of the cells received the same medium with the addition of 100 μg of cycloheximide per ml. After incubation for another hr at 37 C, RNA from control and cycloheximide-treated cells was extracted at room temperature with 0.5% sodium dodecyl sulfate and phenol (20). Samples containing 50 μliters of ^{3}H labeled RNA and 5-μliters of SFV RNA labeled with ^{32}P between 1 and 6 hr postinfection (marker) were subjected to polyacrylamide gel electrophoresis for 4.25 hr on composite 2.0% polyacrylamide-0.5% agarose gels. The gels were processed as described previously (20). Values for ^{3}H were corrected for 5% crossover of ^{32}P, and the data were plotted by computer as percentage of total radioactivity (counts/min) recovered on the gels. The values for total radioactivity were as follows: A, Control, ^{3}H = 12,007 counts/min; ^{32}P = 26,744 counts/min. B, Cycloheximide, ^{3}H = 12,178 counts/min; ^{32}P = 29,785 counts/min. ^{3}H (●); ^{32}P (○). Abbreviations: RI, replicative intermediate; RF, replicative forms.*

Biogenesis of CPV-1 correlated well with the replication of infectious virus, and the time of their appearance in chicken cells was coincident with the reported peaks of SFV and Sindbis RNA synthesis (3, 21, 35). Experiments in which virus replication was inhibited by guanidine or cycloheximide resulted in a decrease or absence of CPV-1. The central question posed by high-resolution autoradiography and cell fractionation experiments summarized by Friedman et al. (accompanying paper) is whether CPV-1 represent the membrane-associated viral replication complex. Conceivably, the clusters of membranous spherules within CPV-1 might be analogous to the factories of other virus infections, in which parental strands of viral nuclei acid are amplified in a specialized environment (7, 30). Friedman and Sreevalsan (14) have shown that 36% of input SFV RNA binds to cell membranes within 1 hr after infection, and it will be of interest to determine whether any of this RNA remains associated with membranes of CPV-1. Efforts to localize replicative RNA by immunologic techniques (32) are in progress.

Temperature-sensitive arbovirus mutants could provide an alternative approach to investigating the relationship of CPV-1 and viral RNA synthesis and would avoid unknown secondary effects of inhibitors on viral development. In some studies already reported (33), temperature-sensitive (RNA) mutants of SFV caused formation of "membranous spherules" (CPV-1) in cells infected at the permissive temperature (30 C), but these structures were fewer, or absent, at the nonpermissive temperature (38.5 C). The SFV mutants made only 13 to 24% of the normal amount of viral RNA, and there was an increased proportion of ribonuclease-resistant (replicative) forms (34). If the temperature-sensitive protein actually represents an RNA polymerase, as suggested by Tan et al. (34), the observed decrease of CPV-1 at nonpermissive temperatures would strongly support other evidence of their role in arbovirus replication.

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LITERATURE CITED

1. Acheson, N. H., and I. Tamm. 1967. Replication of Semliki Forest virus: an electron microscopic study. Virology 32: 128–143.
2. Amako, K., and S. Dales. 1970. Cytopathology of mengovirus infection. II. Proliferation of membranous cisternae. Virology 30:203–213.
3. Ben-Ishai, Z., N. Goldblum, and Y. Becker. 1968. The intracellular site and sequence of Sindbis virus replication. J. Gen. Virol. 2:365–375.
4. Berezesky, I. K., P. M. Grimley, S. A. Tyrrell, and A. S. Rabson. 1971. Ultrastructure of a rat cytomegalovirus. Exp. Mol. Pathol. 14:337 349.
5. Bignami, A., and H. B. Parry. 1971. Aggregations of 35-nanometer particles associated with neuronal cytopathic changes in natural scrapie. Science 171:389–390.
6. Caliguiri, L. A., and I. Tamm. 1970. Characterization of poliovirus-specific structures associated with cytoplasmic membranes. Virology 42:112–122.
7. Dales, S. 1963. The uptake and development of vaccinia virus in strain L cells followed with labeled viral deoxyribonucleic acid. J. Cell Biol. 18:51–72.
8. Ericcson, J. L. E., and B. F. Trump. 1965. Observations on the application to electron microscopy of the lead phosphate technique for the demonstration of acid phosphatase. Histochemie 4:470–487.
9. Friedman, R. M. 1968. Protein synthesis directed by an arbovirus. J. Virol. 2:26–32.
10. Friedman, R. M. 1970. Basis for variable response of arboviruses to guanidine treatment. J. Virol. 6:628–636.
11. Friedman, R. M., and I. K. Berezesky. 1967. Cytoplasmic fractions associated with Semliki Forest virus ribonucleic acid replication. J. Virol. 1:374–383.
12. Friedman, R. M., and P. M. Grimley. 1969. Inhibition of arbovirus assembly by cycloheximide. J. Virol. 4:292–299.
13. Friedman, R. M., H. B. Levy, and W. B. Carter. 1966. Replication of Semliki Forest virus. Three forms of viral RNA produced during infection. Proc. Nat. Acad. Sci. U.S.A. 56:440–446.
14. Friedman, R. M., and T. Sreevalsan. 1970. Membrane binding of input arbovirus ribonucleic acid: effect of interferon or cycloheximide. J. Virol. 6:169–175.
15. Grimley, F. M., I. K. Berezesky, and R. M. Friedman. 1968. Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis. J. Virol. 2: 1326–1338.
16. Grimley, P. M., and R. M. Friedman. 1970. Arboviral infection of voluntary striated muscles. J. Infect. Dis. 122:45–52.
17. Grimely, P. M., and R. M. Friedman. 1970. Development of Semliki Forest virus in mouse brain. Exp. Mol. Pathol. 12:1–13.
18. Huang, A. S., and D. Baltimore. 1970. Defective viral particles and viral disease processes. Nature (London) 226: 325–327.
19. Kovak, W., C. Kung, and L. Stockinger. 1962. Die elektronenmikroskopische Darstellung des Virus der Frühsommer-Meningoencephalitis (FSME) in HeLa Zellen. Arch. Gesamte Virusforsch. 11:544–567.
20. Levin, J. G., and R. M. Friedman. 1971. Analysis of arbovirus ribonucleic acid forms by polyacrylamide gel electrophoresis. J. Virol. 7:504–514.
21. Mecs, E., J. A. Sonnabend, E. M. Martin, and K. H. Fantes. 1967. The effect of interferon on the synthesis of RNA in chick cells infected with Semliki Forest virus. J. Gen. Virol. 1:24–40.
22. Morgan, C., C. Howe, and H. M. Rose. 1961. Structure and development of viruses as observed in the electron microscope. V. Western equine encephalomyelitis virus. J. Exp. Med. 113:219–234.
23. Morgan, C., C. H. Konrad, and H. M. Rose. 1956. Structure and development of viruses as observed in the electron microscope. VII. Incomplete influenza virus. J. Exp. Med. 116:553–564.
24. Murphy, F. A., and S. G. Whitfield. 1970. Eastern equine encephalitis virus infection: electron microscopic studies of mouse central nervous system. Exp. Mol. Pathol. 13:131–146.
25. Mussgay, M., and R. Rott. 1964. Studies on the structure of a hemagglutinating component of a group A arbovirus (Sindbis). Virology 23:573–581.

26. Mussgay, M., and J. Weibel. 1962. Electron microscopic and biological studies on the growth of Venezuelan equine encephalitis virus. Virology 16:52–62.
27. Penman, S. 1965. Stimulation of the incorporation of choline in poliovirus-infected cells. Virology 25:148–152.
28. Pfefferkorn, E. R., and H. S. Hunter. 1963. The source of the ribonucleic acid and phospholipid of Sindbis virus. Virology 20:446–456.
29. Scheele, C. M., and E. R. Pfefferkorn. 1969. Inhibition of interjacent ribonucleic acid (26S) synthesis in cells infected by Sindbis virus. J. Virol. 4:117–122.
30. Silverstein, S. C., and S. Dales. 1968. The penetration of reovirus RNA and initiation of its genetic function in L-strain fibroblasts. J. Cell Biol. 36:197–230.
31. Skinner, M. S., S. Halperen, and J. C. Harkin. 1968. Cytoplasmic membrane-bound vesicles in echovirus 12-infected cells. Virology 36:241–253.
32. Stollar, B. D., and V. Stollar. 1970. Immunofluorescent demonstration of double-stranded RNA in the cytoplasm of Sindbis virus-infected cells. Virology 42:276–280.
33. Tan, K. B. 1970. Electron microscopy of cells infected with Semliki Forest virus temperature-sensitive mutants: correlation of ultrastructural and physiological observations. J. Virol. 5:632–638.
34. Tan, K. B., J. F. Sambrook, and A. J. D. Bellett. 1969. Semliki Forest virus temperature-sensitive mutants: isolation and characterization. Virology 38:427–439.
35. Taylor, J. 1965. Studies on the mechanism of action of interferon. Virology 25:340–349.
36. Waite, M. R. F., and E. R. Pfefferkorn. 1970. Phospholipid synthesis in Sindbis virus-infected cells. J. Virol. 6:637–643.
37. Weibel, E. R. 1963. Principles and methods for the morphometric study of the lung and other organs. Lab. Invest. 12: 131–155.