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THE STRUCTURE AND REPLICATION OF
SEMLIKI FOREST VIRUS,
A MOSQUITO-BORNE ANIMAL VIRUS

A thesis submitted to the Faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

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Approved for Publication
Igor Tamm
Professor

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SUMMARY

Semliki Forest virus is a group A arthropod-borne virus which contains RNA and possesses a lipoprotein envelope. It can be transmitted among animals by mosquitoes in which it multiplies, but its natural host is not known. The virus grows to high titer in a number of types of vertebrate cells in culture.

The growth of Semliki Forest virus was studied with the electron microscope during a single cycle of viral replication in chick embryo cells. The spherical virus particle consists of a nucleoid, or nucleocapsid, 280 A in diameter, closely wrapped in an envelope which consists of a unit membrane 75 A thick coated on its outer surface with projections 110 A long. There appears to be a narrow 10-30 A space between the nucleocapsid and the envelope. The diameter of the virus particle, excluding the projections, is approximately 500 A, and thus the whole virus particle is about 700 A in diameter.

After a latent period of 2 to 3 hours, infectious virus is produced at a nearly constant rate of approximately 200 plaque-forming units/cell per hour, until the 9th hour. During the period of rapid production of virus, individual free virus nucleocapsids are found scattered in the cytoplasmic matrix. The nucleocapsids appear to migrate singly to the plasma membrane, or to the membranes of certain vacuoles, where complete virus particles are formed by a budding process. An envelope, consisting of a portion of the membrane covered with projections, encloses the nucleocapsid as it is extruded into the extracellular space or the interior of a vacuole. Most developing virus particles are seen at the plasma membrane; fewer are found at the membranes of vacuoles.

Small vesicles, consisting of buds of membrane, are found projecting into large cytoplasmic vacuoles or into the extracellular space at the plasma membrane. These vesicles do not contain nucleocapsids, and there is little evidence of the projections which virus envelopes have. The exact nature of the vesicles is unknown, but they may represent aberrant envelopes.

Late in the growth cycle, nucleocapsids are increasingly found lining the cytoplasmic surface of round or tubular vacuoles. These vacuoles often contain long cylindrical structures which appear to be formed from a membrane covered with amorphous material. Because the vacuoles lined with nucleocapsids appear in large numbers only at late stages in the viral growth cycle, they do not appear to have a functional role in the production of Semliki Forest virus particles.

Nucleocapsids of Semliki Forest virus have been isolated and purified from homogenates of infected chick embryo cells. The purification procedure consists of a preliminary centrifugation in a sucrose gradient, followed by dialysis against a buffer lacking magnesium. Ribosomes contaminating the nucleocapsids are dissociated into subunits by this means. The dialyzed material is resedimented twice in succession in sucrose gradients, to separate the nucleocapsids from the ribosomal subunits and to concentrate the purified nucleocapsids.

Purified, negatively-stained nucleocapsids are roughly spherical and 400 Å in diameter, slightly larger than nucleocapsids in thin sections (280 Å). The reason for this discrepancy is not known. Nucleocapsids are penetrated by the negative stain (phosphotungstate) in some preparations but not in others. A mottled surface structure indicates that the nucleocapsids may have a subunit structure, but there is no clear indication of symmetry.

Unfixed nucleocapsids disintegrate in high concentrations of cesium chloride, but after formaldehyde fixation they can be banded to an equilibrium density of 1.47 gm/cc in cesium chloride gradients. In potassium tartrate gradients nucleocapsids band at an equilibrium density of 1.34 gm/cc, but the RNA within nucleocapsids is fragmented by this procedure. The high buoyant density and the ultraviolet absorbance profile of nucleocapsids indicate an RNA content greater than 30% of the total mass.

Nucleocapsids contain 45 S RNA, found also in complete virus. Two protein bands are resolved on gel electropherograms of purified nucleocapsids. The major protein has a molecular weight of approximately 35,000, and the minor protein, 70,000. The latter protein is

present only in small amounts, and may be a dimer of the major protein. Purified virus contains these two proteins, in the same proportion as in the nucleocapsids, and, in addition, a major protein of molecular weight approximately 55,000. This protein is probably located in the virus envelope.

The RNA within nucleocapsids is accessible to digestion by pancreatic ribonuclease. The nucleocapsids differ in this feature from most small RNA-containing viruses, which are resistant to ribonuclease. The nucleocapsid structure disintegrates upon digestion of the RNA by ribonuclease.

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I. GENERAL INTRODUCTION

A. Semliki Forest Virus, an Arthropod-Borne Animal Virus

Semliki Forest virus was first isolated from mosquitoes collected from humans near the Semliki Crown Forest in western Uganda in 1942 (Smithburn and Haddow, 1944). The virus was isolated by injecting a filtered homogenate of 130 female Aedes abnormalis mosquitoes intracerebrally into several adult white mice, one of which subsequently became ill. The virus was then passaged in mice by intracerebral injection of clarified, filtered mouse brain suspensions. After several passages inoculated mice became ill with encephalitis and died within 48 hours after infection. Several other mammals, including a number of primates, became ill and died, often after suffering paralysis, when inoculated intracerebrally.

Subsequent immunological surveys (Smithburn et al., 1944) demonstrated circulating antibodies against Semliki Forest virus in a number of wild-caught monkey species, and in from 5 to 30 percent of humans tested from various areas of Uganda. Thus it appears to be a virus which commonly infects humans and nonhuman primates, although no human disease has been associated with the virus. More recently, an immunologically identical virus has been isolated from mosquitoes in Cameroon (Macnamara, 1953) and in Mozambique (McIntosh et al., 1961), indicating that it has a wide distribution within Africa. Serological studies (Smithburn et al., 1954) suggest that the virus may also occur in Malaya and North Borneo.

Semliki Forest virus can be transmitted from one animal to another by the bite of one of several species of mosquitoes (Collins, 1963; Davies and Yoshpe-Purer, 1954; Nye and Lien, 1960; Nye and Bertram, 1960; Woodall and Bertram, 1959). It has been shown (Mims et al., 1966) that the virus multiplies in the mosquito, especially in the salivary glands from which it is injected into the vertebrate host. Thus it is a member of the large group of viruses known as the arthropod-borne viruses, a name usually abbreviated to arboviruses. The group includes such pathogens as dengue, yellow fever, St. Louis encephalitis and Russian spring-summer encephalitis virus. To date, some 300 antigenically distinct arboviruses, transmitted by mosquitoes, ticks, or sandflies, have been isolated. These have been classed into

four main groups (A, B, C, and Bunyamwera), and a number of minor groups on the basis of immunological relatedness (Casals and Reeves, 1965). In addition, there are a number of arthropod-borne viruses which remain ungrouped.

The arbovirus group is thus a large, medically and biologically important family of animal viruses. Semliki Forest virus, a member of subgroup A, has several characteristics which make it a favorable model for the study of arbovirus structure and biochemistry. It grows to high titer in a variety of cell types in culture (Henderson and Taylor, 1960), is easily assayed by either plaque formation (Cheng, 1961), or hemagglutination (Clarke and Casals, 1958), and is not pathogenic for man. Recently, a number of temperature-sensitive mutants have been isolated (Sambrook, 1965), making it possible to determine the functions of the viral genetic material by combined biochemical and genetic techniques. In all these respects, Semliki Forest virus is quite similar to Sindbis virus, another group A arbovirus with which much experimental work has been carried out in the past few years (Burge and Pfefferkorn, 1966a, 1966b; Pfefferkorn and Hunter, 1963; Mussgay and Rott, 1964).

B. Virus Structure and Classification

The arbovirus group has been defined by a biological and ecological characteristic; that is, transmission of virus between vertebrate hosts via an arthropod vector. However, most other viruses of vertebrates have been conveniently grouped on the basis of the chemical and morphological characteristics of the virus particles. To place the arboviruses into this taxonomic context, I will briefly discuss virus structure and the classification of viruses of vertebrates.

The currently accepted classification of animal viruses (Fenner, 1968) is based on three essential features of virus particles: (1) Type of nucleic acid; (2) structure of the protein coat or capsid; (3) presence or absence of a lipid-containing envelope. These features are illustrated in the diagram of virus particles in Fig. 1. Let us consider each element in detail.

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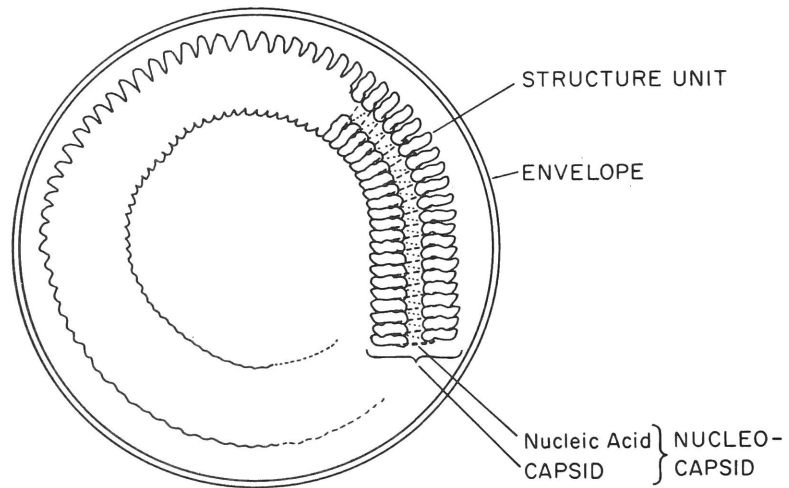
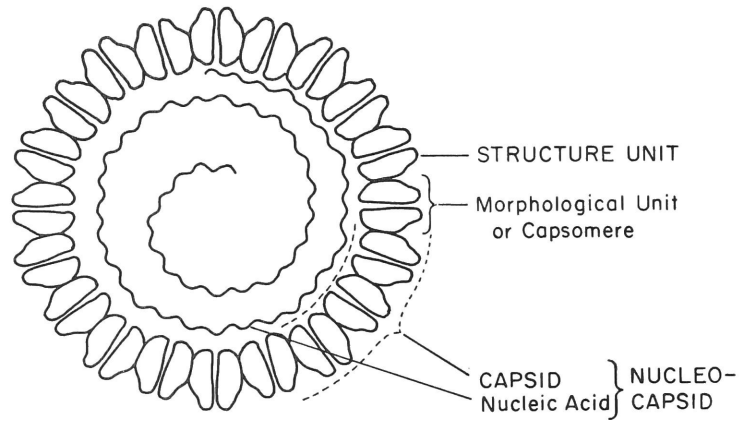
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Fig. 1. Diagram illustrating the components of animal virus particles. The upper figure represents an unenveloped isometric virus particle; the representation of the nucleic acid is purely diagrammatic and is not intended to correspond to the actual distribution of RNA within the nucleocapsid. The lower figure represents an enveloped virus particle with a helical nucleocapsid.

To face page 4.



All viruses contain nucleic acid as their genetic material: this can be either RNA or DNA, but so far no virus has been found which contains both RNA and DNA, and this exclusivity has become part of the definition of a virus. The nucleic acid can be either single stranded or double stranded.

The nucleic acid is associated with a protein coat, or capsid, constructed from a number of protein subunits. These subunits, called "structure units," are symmetrically arranged about the nucleic acid; the nucleic acid plus capsid is called "nucleocapsid." The capsid has either helical symmetry, as in a spiral, or icosahedral symmetry, a complex type of cubic symmetry. Icosahedral nucleocapsids are closed, roughly spherical shells. The structure units are usually grouped on the surface of icosahedral capsids into morphologically distinct clusters called "morphological units" or "capsomeres." Groups of animal viruses are distinguished on the basis of the symmetry of the capsid, its dimensions, and the number and arrangement of structure units.*

The envelope, if present, encloses the nucleocapsid. It contains lipid, protein, and probably carbohydrate. At least some of the envelope proteins are virus-specific. The envelope is morphologically similar to a cellular membrane, but possesses in addition a fringe of projections on its outer surface. These projections may consist of protein, carbohydrate, or both. The possession or lack of an envelope is a feature used to distinguish groups of animal viruses.

Most animal viruses have been classified into a number of major groups on the basis of these chemical and structural criteria (Table I): for example, the picornaviruses have single-stranded RNA, an icosahedral capsid symmetry, and no envelope; the adenoviruses have double-stranded DNA, icosahedral symmetry, and no envelope; the myxoviruses have single-stranded RNA, helical symmetry, and possess an envelope. The arboviruses, as might be expected, do not fit neatly into this scheme. The arthropod-borne viruses are heterogeneous with respect to size, capsid structure,

* It should be noted that some viruses may have a second protein coat (e.g. reovirus) or internal proteins (e.g. adenoviruses) in addition to the outer capsid.

TABLE I
 Characteristics of Major Groups of Animal Viruses
 (Adapted from Fenner, 1968)

	Strandedness of nucleic acid	Size of virus particles, nm	Presence of envelope	Capsid symmetry	Number of structure units or helix diameter
<u>RNA Viruses:</u>					
Picornavirus	SS	20-30	-	Icosahedral	?
Reovirus	DS	60-90	-	Icosahedral	540
Encephalovirus ^a	SS	50-80	+	?	?
Rhabdovirus	SS	70x180	+	Helical	5 nm
Myxovirus	SS	80-120	+	Helical	8-9 nm
Paramyxovirus	SS	120-450	+	Helical	17-18 nm
Leukovirus	SS	100-120	+	?	?
<u>DNA Viruses:</u>					
Parvovirus	SS	20	-	Icosahedral	180
Adeno-associated virus	DS	20	-	Icosahedral	?
Papovavirus	DS	30-50	-	Icosahedral	420
Adenovirus	DS	80-90	-	Icosahedral	1500
Herpesvirus	DS	100-150	+	Icosahedral	960
Poxvirus	DS	200-300	+	Complex	?

^a Includes most arthropod-borne viruses (arboviruses).

and possession of an envelope. However, if only a relatively small number of types are left out, most viruses in the arbovirus group do fit into what appears to be a single class. Certainly this class includes all of the group A and B arboviruses; many other arboviruses appear to be similar in structure to group A and B viruses. Viruses in this class are sensitive to lipid solvents, such as ether and sodium deoxycholate (Andrewes and Horstman, 1948; Theiler, 1957) and thus probably possess an envelope; contain single-stranded RNA, which in a number of cases has been successfully extracted in infectious form (Cheng, 1958; Wecker, 1959); and are approximately spherical and of relatively uniform size (500-800 A diameter) (Cheng, 1961; Morgan et al., 1961; Sharp et al., 1943). However, the symmetry of the capsid has not been conclusively determined for any arbovirus.

It has been suggested (Fenner, 1968) that the arboviruses which share these characteristics be grouped together under the name "encephaloviruses," a name which refers to the fact that many of them cause encephalitis. Vesicular stomatitis virus, a bullet-shaped arthropod-borne virus, would then be grouped with the numerous other bullet-shaped viruses which have been recognized (rhabdoviruses); and bluetongue virus would be grouped with the structurally similar reoviruses. This suggested reclassification of a large group of important viruses would begin to clarify the similarities, and differences, among the arthropod-borne viruses. I shall now discuss the chemistry, structure and biochemistry of replication of group A arboviruses, as an introduction to the data to be presented in the body of this thesis.

C. Characteristics of Group A Arboviruses

1. Chemistry

The most detailed information on the chemical composition of a group A arbovirus comes from Pfefferkorn and Hunter (1963), who analyzed purified Sindbis virus preparations. Virus was purified by adsorption to and elution from aluminum phosphate, followed by differential centrifugation. Analysis by colorimetric methods indicated that virus particles contain 65% protein, 28% lipid, and 6% RNA. Carbohydrate analysis

was not attempted. Cholesterol accounts for 25% of the lipid and phospholipids for the remaining 75%. Sphingomyelin represents one quarter of the phospholipid in the virus, although it is only 11% of the phospholipid of the host chick embryo cells.

Thus Sindbis virus has a protein to lipid ratio of approximately 2.3:1, and a high sphingomyelin content. The latter is most likely explained by the recent findings that plasma membranes of many vertebrate cells have much higher sphingomyelin contents than the remainder of the cellular membranes (Klenk and Choppin, 1969; Skipski et al., 1965). Most Sindbis virus particles probably acquire their envelopes at the plasma membrane of the cell.

Pfefferkorn and co-workers also showed that while the RNA and protein of Sindbis virus are newly synthesized during the growth of the virus in chick embryo cells, the phospholipids present in the virus are derived from pre-existing cellular phosphatides (Pfefferkorn and Hunter, 1963; Pfefferkorn and Clifford, 1964). The implication of these results was that the viral envelope is formed by a combination of cellular lipids with newly synthesized viral proteins. This was largely confirmed by more recent analysis of Sindbis (Strauss et al., 1968), western equine encephalitis (Sreevalsan and Allen, 1968), and Semliki Forest virus (Friedman, 1968b; Hay et al., 1968) proteins, which showed that the viral envelope contains only one polypeptide chain as determined by electrophoretic migration in polyacrylamide gels. It was also reported that the nucleocapsid of Sindbis virus contains only one electrophoretically distinguishable protein (Strauss et al., 1968). However, two proteins have been found in Semliki Forest virus nucleocapsids analyzed by gel electrophoresis (Friedman, 1968b).

Group A arbovirus particles contain single-stranded RNA which sediments at 40 to 45 S, depending on the virus type and the exact conditions of sedimentation. The RNA is ribonuclease-sensitive, and contains unequal amounts of the four ribonucleotides. The molecular weight of the RNA of group A arboviruses is not known, but it probably falls within a range of 2-5 million daltons. A recent report (Strauss et al., 1969) refers to unpublished findings that the RNA of Sindbis virus has a molecular weight of 4 to 5 million daltons.

2. Morphology

In an early morphological study of Semliki Forest virus (Cheng, 1961), electron micrographs of chromium-shadowed virus revealed collapsed spherical particles of a relatively uniform diameter. Electron micrographs of negatively-stained Sindbis virus (Mussgay and Rott, 1964) and Semliki Forest virus (Osterreith and Calberg-Bacq, 1966) showed that particles consisted of an envelope with a border of thin projections, surrounding a spherical core. The envelopes could be removed by treatment with a protease, caseinase C (Osterreith and Calberg-Bacq, 1966). "Empty" envelopes could be isolated from crude virus preparations by cesium chloride density gradient centrifugation (Mussgay and Rott, 1964). In both cases, it was shown that the viral hemagglutinin was associated with the envelope, rather than the core. Other group A and group B arboviruses have a similar morphology (Wecker and Richter, 1962; Saturno, 1963; Klimenko et al., 1965; Kitaoka and Nishimura, 1963; Simpson and Hauser, 1968a).

Thin sections of cells infected with certain group A and group B arboviruses had been examined by several investigators prior to the present study (Chain et al., 1966; Morgan et al., 1961; Mussgay and Weibel, 1962; Ota, 1965). Morgan et al. (1961) examined by electron microscopy the growth of western equine encephalitis virus, a group A arbovirus, in several cell types in tissue culture. They found "precursor particles," which probably correspond to the viral nucleocapsid, lining the cytoplasmic side of certain vacuoles in infected cells. It was suggested that the nucleocapsids are assembled at template sites close to the vacuolar membranes, and are incorporated into mature virus particles by "budding" across cellular membranes.

3. Biochemistry of Replication

Very little is known about the early stages of the infection of susceptible cells by group A arboviruses. Adsorption to the cell surface seems to be rapid (Dulbecco and Vogt, 1954), and penetration probably takes place via phagocytosis of virus particles (Grimley et al., 1968) as appears likely for several other enveloped and non-enveloped viruses (Dales, 1965). It is not known whether the viral nucleocapsid

is released intact into the cytoplasm and subsequently releases the viral RNA, or if the RNA is released directly into the cytoplasmic matrix from a phagocytic vesicle. It is clear that infection can be initiated by the viral RNA alone; infectious RNA has been isolated from a number of group A arboviruses (Cheng, 1958; Wecker, 1959).

a. Viral protein synthesis. In cells infected with a picornavirus such as poliovirus, it is well established that most virus-specific protein synthesis takes place on polyribosomes which are bound to intracellular membranes (Penman et al., 1964). Host cell polyribosomes, most of which in HeLa cells are not associated with membranes, are rapidly degraded, and large polysomes containing poliovirus RNA are subsequently found in the membrane fraction of cytoplasmic extracts from infected cells (Becker et al., 1963; Penman et al., 1964). These polysomes can be released by detergent treatment, which solubilizes the lipoprotein membranes. Newly-synthesized viral protein is found associated with the polysomes (Scharff et al., 1963).

Friedman (1968a) and Ben-Ishai et al. (1968) claim that protein synthesis in chick embryo cells infected with Semliki Forest virus or with Sindbis virus also takes place in association with membranes. Although work from both laboratories shows that viral protein synthesis takes place on structures which sediment faster than 200-300 S, no conclusive evidence has been reported that these structures represent membrane-attached polyribosomes.

An RNA polymerase which produces virus-specific RNA is synthesized in Semliki Forest virus-infected chick cells (Martin and Sonnabend, 1967). This polymerase is present predominantly in the large-particle fraction of cytoplasmic extracts; presumably the enzyme is membrane-bound, although again no conclusive evidence on this point has been reported.

Hay et al. (1968) analyzed the proteins in fractions of cell extracts by gel electrophoresis at various times after Semliki Forest virus infection. They found that at least six new proteins are synthesized in infected cells; at no time are any of these proteins present in the soluble phase of the cytoplasm. Thus viral proteins are at all

times either bound to heavy structures, possibly cellular membranes, or assembled in particulate form, probably as complete nucleocapsids and virus particles.

Much experimental work remains to be done to characterize arbovirus protein synthesis; however, in analogy with the better-studied picornaviruses, it appears likely that the polysomes which translate virus messenger RNA into virus proteins are associated with large structures, most likely membranes, in the cell cytoplasm. These proteins do not seem to be released after synthesis, but either remain associated with large structures or rapidly enter virus nucleocapsids and virus particles.

b. RNA synthesis. Three types of RNA are found in cells infected with group A arboviruses (Friedman et al., 1966; Sreevalsan and Lockart, 1966; Sonnabend et al., 1967): they have approximate sedimentation coefficients of 45 S, 26 S, and 20 S. The 45 S form is single stranded and corresponds to the RNA in mature virus. The 26 S RNA is ribonuclease-sensitive (Friedman et al., 1966) and has a base composition and buoyant density (Friedman and Berezesky, 1967; Sreevalsan et al., 1968) similar to that of the 45 S RNA. Thus it is also single stranded. It has a much lower specific infectivity than 45 S RNA (Sonnabend et al., 1967; Sreevalsan and Lockart, 1966). Sreevalsan et al. (1968) report interconversion of 45 S and 26 S RNA after denaturation or change of salt concentration. However this is difficult to distinguish from partial breakage and nonspecific aggregation of RNA molecules.

The 20 S RNA is partly ribonuclease resistant (Friedman et al., 1966; Sonnabend et al., 1967). It has a very high melting temperature (103 C) and becomes completely ribonuclease-sensitive if melted and rapidly cooled (Sonnabend et al., 1967). In addition, it has a lower buoyant density than the single-stranded 45 S species (Martin and Sonnabend, 1967). These characteristics all indicate that the 20 S RNA is a double-stranded form.

The 20 S form is the first RNA to be labeled by a short pulse of ³H-uridine given early in the period of viral RNA synthesis. The 26 S RNA takes up label somewhat more slowly, and the 45 S form is labeled

only after a delay of some 15-30 minutes (Friedman et al., 1966). However, the 20 S RNA is never present in large amounts in infected cells (Mecs et al., 1967). The 26 S RNA is the major species present at all times in cells infected with western equine encephalitis virus (Sreevalsan and Lockart, 1966), while in Semliki Forest virus-infected cells, the 45 S RNA is the predominant form late in the infectious cycle (Mecs et al., 1967).

The kinetics of labeling of the various RNA's imply that the 20 S RNA, or a part of it, is a precursor to the 26 S and 45 S forms. The 20 S form thus may be analogous to the "replicative intermediate" found in bacterial cells infected with RNA phages. Three kinds of evidence reinforce this hypothesis. First, the infecting RNA enters a 20 S form soon after infection (Friedman et al., 1966; Pfefferkorn et al., 1967), suggesting that the 20 S RNA is a duplex consisting of the infecting "plus" strand and a newly-synthesized "minus" strand. Second, a viral RNA polymerase from virus-infected cells synthesizes, in vitro, 20 S RNA which is indistinguishable from the 20 S RNA found in infected cells (Martin and Sonnabend, 1967). Third, the base composition of 20 S RNA synthesized early in the infectious cycle resembles that expected of a duplex: the 20 S RNA synthesized later has the base composition of the "plus" (45 S) viral RNA strand (Friedman and Berezesky, 1967; Kaariainen and Gomatos, 1969). This suggests that at early times, both strands are being synthesized, but that later only the plus strands are made, using the previously synthesized minus strands as template.

Cell fractionation studies (Kaariainen and Gomatos, 1969) show that the 20 S RNA is located in a large particle fraction which is probably membranous, and the RNA can be released by treatment with sodium deoxycholate. Some 26 S RNA is also found in this fraction. However, the 45 S RNA is never found associated with membranes; it is found in nucleocapsids and completed virus particles. Not all of the 26 S RNA is present in the large particle fraction (Kaariainen and Gomatos, 1969); according to one report (Sreevalsan and Allen, 1968), a 65 S particle, whose integrity is destroyed by EDTA or RNase treatment, also contains 26 S viral RNA. This may be a precursor to,

or a breakdown product of, viral polyribosomes. It may also be an artifactual combination of cellular protein with free viral RNA, as is found in extracts of poliovirus-infected cells (Girard and Baltimore, 1966). The type of RNA present in viral polyribosomes has not been directly determined.

To summarize, the 20 S RNA is probably a replicative form of viral RNA, and it is found exclusively associated with membranous structures, where the viral RNA polymerase is located. The 26 S RNA has an unknown function, although it may act as a messenger for the translation of virus proteins. This may or may not take place on membranes. The 45 S RNA, which is the most highly infectious of the three types, is incorporated into nucleocapsids and completed virus particles and is thus the progeny viral RNA.

II. AN ELECTRON MICROSCOPIC STUDY
OF THE REPLICATION OF SEMLIKI FOREST VIRUS
IN CHICK EMBRYO CELLS

A. Introduction

Semliki Forest virus is a favorable model for the study of the structure and assembly of group A arboviruses. It grows well in several types of cells in culture, is easily assayed by plaque formation, and is not pathogenic for man. A previous electron microscopic study of the development of western equine encephalitis virus, another group A arbovirus, demonstrated that mature virus is formed by the budding of "precursor particles" or virus nucleoids across cellular membranes (Morgan et al., 1961). Nucleoids were also found lining the cytoplasmic surface of certain membrane-bounded vacuoles. It was suggested that the nucleoids are assembled at template sites close to these vacuolar membranes.

In the present investigation, the replication of Semliki Forest virus during a single cycle of growth was studied with the electron microscope. Morphological findings were correlated with the kinetics of production of infectious virus. Evidence was obtained that the envelope, which surrounds the viral nucleoid during virus assembly, consists of a portion of a cellular membrane which has become covered with projections. Small empty vesicles were found apparently budding from the plasma membrane or the inner surface of certain vacuoles. These vesicles may represent aberrant viral envelopes. In addition it was found that the aggregation of nucleoids around cytoplasmic vacuoles is a phenomenon that occurs late in the virus growth cycle and is probably not related to the assembly of nucleoids.

B. Materials and Methods

1. Virus

The Kumba strain of Semliki Forest virus (Macnamara, 1953; Smithburn, 1952; Kerr, 1952) was obtained from Dr. Robert M. Friedman through the courtesy of Dr. Charles E. Buckler, Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases. It was grown at 37 C in primary cultures of chick embryo cells or in a continuous line of baby hamster kidney cells (BHK21) obtained from Dr. N. Karabatsos, Rockefeller Foundation Virus Laboratory. Growth medium was reinforced Eagle's medium (Bablanian et al., 1965) with

0.5% bovine serum albumin. When cytopathic effects were maximal, cells were scraped from the petri dish, frozen with the medium and rapidly thawed, and the cellular debris was sedimented at 1000 x g for 15 minutes at 4 C. The supernatant fluid containing the virus was stored at -56 C.

The virus employed had undergone several passages in chick embryo cells in this laboratory, and produces both small and large plaques. The experiments reported here were carried out with stocks containing about 90% small plaque (1 to 3 mm) and 10% large plaque (4 to 6 mm) virus. It has thus far been possible to isolate and prepare pure stocks of the small plaque variant, but not of the large plaque variant.

2. Cell Cultures

All experiments were carried out in primary monolayer cultures of chick embryo cells. 9 to 11-day old embryos were minced and trypsinized. 60 mm plastic petri dishes were seeded at a cell density sufficient to allow growth to a monolayer of 4×10^6 cells in three days at 37 C in lactalbumin hydrolyzate medium with 2% calf serum (Choppin, 1964).

3. Assay of Infectious Virus

Plaque assays were performed on monolayers of chick embryo cells. Cell sheets were washed once with phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) and inoculated with virus diluted in Eagle's medium containing 1% bovine serum albumin. After adsorption for 1 hour at 37 C, the inoculum was removed and 8 ml of agar overlay medium (1.9% agar, 0.1% yeast extract, and 0.1% bovine serum albumin in reinforced Eagle's medium) was added. Plaques, visualized by oblique lighting, were counted after incubation for 44-48 hours at 35 C.

4. Growth Curve

An input multiplicity of 50 plaque-forming units (PFU) per cell was chosen to ensure a single cycle infection. Chick embryo cell monolayers were washed once with PBS and inoculated with Semliki Forest virus in Eagle's medium with 1% bovine serum albumin. After adsorption for 1 hour at 37 C, the inoculum was removed and 2 ml of reinforced Eagle's medium with 0.5% bovine serum albumin was added. Plates were incubated at 37 C and virus was harvested and assayed as described above. Cytopathic changes were viewed in an inverted phase-contrast microscope.

5. Electron Microscopy

Monolayers were inoculated as described above, and incubated at 37 C. At 2-hour intervals from 3 to 17 hours after infection, the cells from several petri dishes were scraped, pelleted, and fixed with 1% glutaraldehyde followed by 1% osmium tetroxide in PBS without antibiotics, according to the procedures of Dales et al. (1965). In some experiments, the cells were released by incubation for several minutes with 0.25% trypsin (Nutritional Biochemicals) and 0.01 M ethylenediamine tetraacetate (EDTA) in PBS lacking Mg^{++} and Ca^{++} , then recombined with the original medium before pelleting. Cells processed in this way were rounded and separated from each other, so that the structures present in each cell profile could be more easily enumerated. In addition, there were fewer disrupted cells than when the cells were harvested by scraping. Micrographs of trypsin-released cells are so identified in the figure legends. Some pellets were stained directly after fixation with 0.5% uranyl acetate in veronal-acetate buffer, pH 5, for 1 or 2 hours at room temperature (Farquhar and Palade, 1965). Micrographs of cells stained in this manner are identified as "stained with uranyl acetate" in the figure legends. All pellets were dehydrated in ethanol and embedded in epoxy resin (Luft, 1961). Sections were cut with a diamond knife and stained by 1-minute applications of saturated uranyl acetate diluted 1:1 with ethanol (Watson, 1959), and 0.4% lead citrate in 0.1 N sodium hydroxide (Venable and Coggeshall, 1965). Specimens were examined in a RCA EMU-3F, a Hitachi HS-7S, or a Siemens Elmiskop I electron microscope.

Negative staining was done by the method of Brenner and Horne (1959). Samples of virus (about 10^9 PFU/ml) were applied to grids covered with formvar and a carbon film. The grids were rinsed with distilled water, and a drop of 1% potassium phosphotungstate, pH 6.2, was applied. The excess phosphotungstate was removed with a piece of filter paper after 30 seconds. The grids were then air-dried and examined in the electron microscope.

B. Results

1. Growth Kinetics of Semliki Forest Virus in Chick Embryo Cells

When inoculated onto monolayer cultures of primary chick embryo cells at a multiplicity of 50 PFU/cell, Semliki Forest virus has a latent period of 2 to 3 hours, and a maximum yield of 2000 PFU/cell is produced by 10 to 12 hours after infection (Fig. 2). An arithmetic plot of yield of infectious virus against time shows that the rate of virus production is constant, about 200 PFU/cell per hour, from the 5th through the 9th hour after infection (Fig. 3). These growth kinetics are similar to those reported by Taylor (1965).

Cytopathic changes begin to appear at about 6 hours after infection (Fig. 2), and by 12 hours most of the cells have rounded up and are detaching from the surface of the Petri dish. Thus the cessation of virus production coincides with the time when cytopathic effects become maximal.

2. Structure of Semliki Forest Virus

Negatively-stained Semliki Forest virus particles (Fig. 4) are roughly circular in profile and have a diameter of 700 to 800 Å, including a border of radial projections 110 Å long. The diameter of the virus particle minus the projections is 500 to 550 Å.

In thin sections from samples stained with uranyl acetate in the pellet (Fig. 5), the internal structure of virus particles is seen to consist of a nucleoid 280 Å in diameter separated by a narrow space from a surrounding triple-layered unit membrane about 75 Å thick. Extending 50 to 100 Å beyond the outer leaflet of the membrane there is seen in some places an irregular, diffusely-staining material which may correspond to the projections more clearly visualized in negatively-stained virus.

Samples not stained with uranyl acetate in the pellet (Fig. 6) do not show the triple-layered unit membrane. In favorable views, the image of the virus particle shows a 280 Å nucleoid which is surrounded by a single dense layer 20-30 Å thick, and a lightly-staining border of variable thickness. A comparison of Figs. 5 and 6 reveals that the

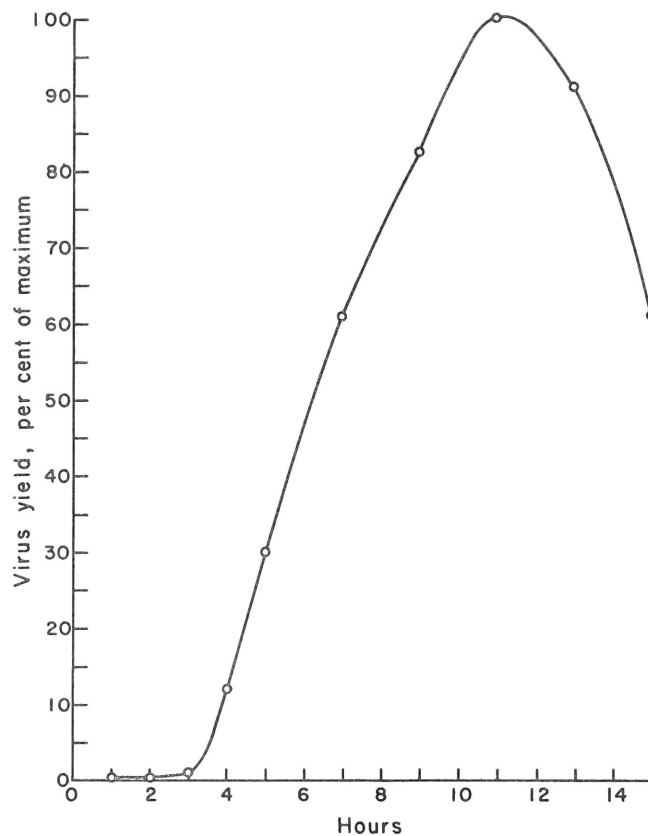


Fig. 2. Growth curve of Semliki Forest virus in chick embryo cells. Monolayers were inoculated at a multiplicity of 50 PFU/cell. Cytopathic effects (CPE), estimated on an arbitrary scale of 0 to +++, consisted of rounding, retraction of cell processes, and detachment of cells from the Petri dish.

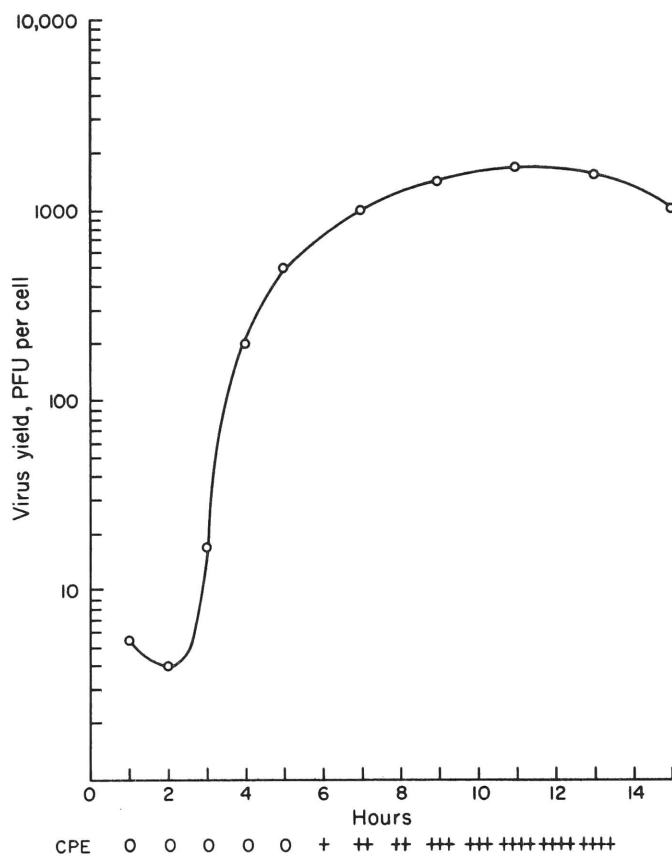
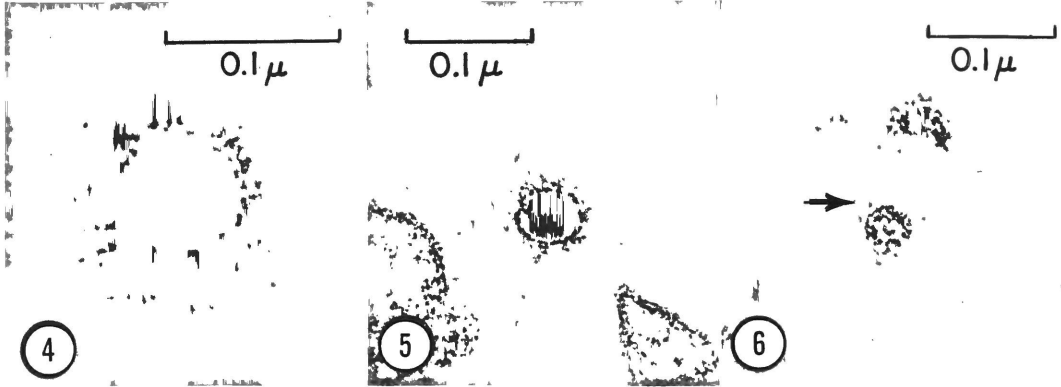


Fig. 3. Arithmetic plot of the growth of Semliki Forest virus in chick embryo cells. Curve based on data also used for Fig. 1.

Fig. 4. Semliki Forest virus negatively stained with phosphotungstate. A border of projections surrounds a nearly spherical body. Magnification: x 225,000.

Fig. 5. Extracellular particle of Semliki Forest virus in a thin section of infected chick embryo cells. Sample stained in the pellet with uranyl acetate before sectioning. Both the inner and the outer leaflets of the unit membrane are visible in cellular membranes as well as in the virus particle. The diffusely-staining material beyond the membrane of the virus particle may represent a portion of the projections on the virus envelope. Magnification: x 165,000.

Fig. 6. Extracellular virus particles in a thin section of infected chick embryo cells. The triple-layered structure of the unit membrane is not revealed in this preparation, which was not stained with uranyl acetate in the pellet. The nucleoid is encircled by what appears to be the inner leaflet of a unit membrane. The outer leaflet is probably located within the lightly-staining area (arrow). Magnification: x 165,000.



single dense layer of Fig. 6 corresponds in thickness and position in the virus particle to the inner leaflet of the unit membrane in Fig. 5. It is likely that the lightly-staining border of Fig. 6 marks the location of the outer leaflet and the sites of insertion of the projections.

We conclude from these three views that virus particles consist of a nucleoid, 280 A in diameter, enclosed in a unit membrane 75 A thick which is coated on its outer surface with projections 110 A long. There appears to be a narrow 10-30 A space between the nucleoid and the unit membrane. The diameter of the virus particle, excluding the projections, is approximately 500 A, and thus the whole virus particle is about 700 A in diameter. The projections, which are clearly visualized in negatively-stained samples, are poorly stained and only incompletely seen in sectioned material.

3. Appearance of Cells Producing Virus at Maximal Rate

Since cells are producing virus at the maximal rate from 5 to 9 hours after infection, all features of virus formation detectable with the electron microscope should be present during this time. A majority of the cells sampled in this interval (Fig. 7) show no striking changes in the morphology of subcellular organelles compared with uninfected chick embryo cells. Mitochondria are well preserved, rough and smooth endoplasmic reticulum is dispersed throughout the cytoplasmic matrix, and the nucleus shows scattered granular material, dense nucleoli, and an intact nuclear envelope.

In the infected cells, virus particles are seen budding from cellular membranes (Fig. 8), and completed particles lie in the extracellular space, or in vacuoles near the cell border. Free virus nucleoids can be seen scattered in the cytoplasmic matrix (Fig. 8), as well as at the plasma membrane. Except for collections of small vesicles which will be described in a later section, there are no other outstanding morphological features in the majority of cells 5 to 9 hours after Semliki Forest virus infection. It should be emphasized that by 5 hours the rate of virus production has reached its maximum, and that approximately 30% of the total virus yield has already been produced.

Fig. 7. A portion of a cell sampled 5 hours after infection. The nucleus (N) contains a dense nucleolus. Mitochondria (M), rough and smooth endoplasmic reticulum, and a centriole (C) are visible in the cytoplasm. Two virus particles (arrows) lie in the extracellular space at an invagination of the plasma membrane. Magnification: x 32,000.

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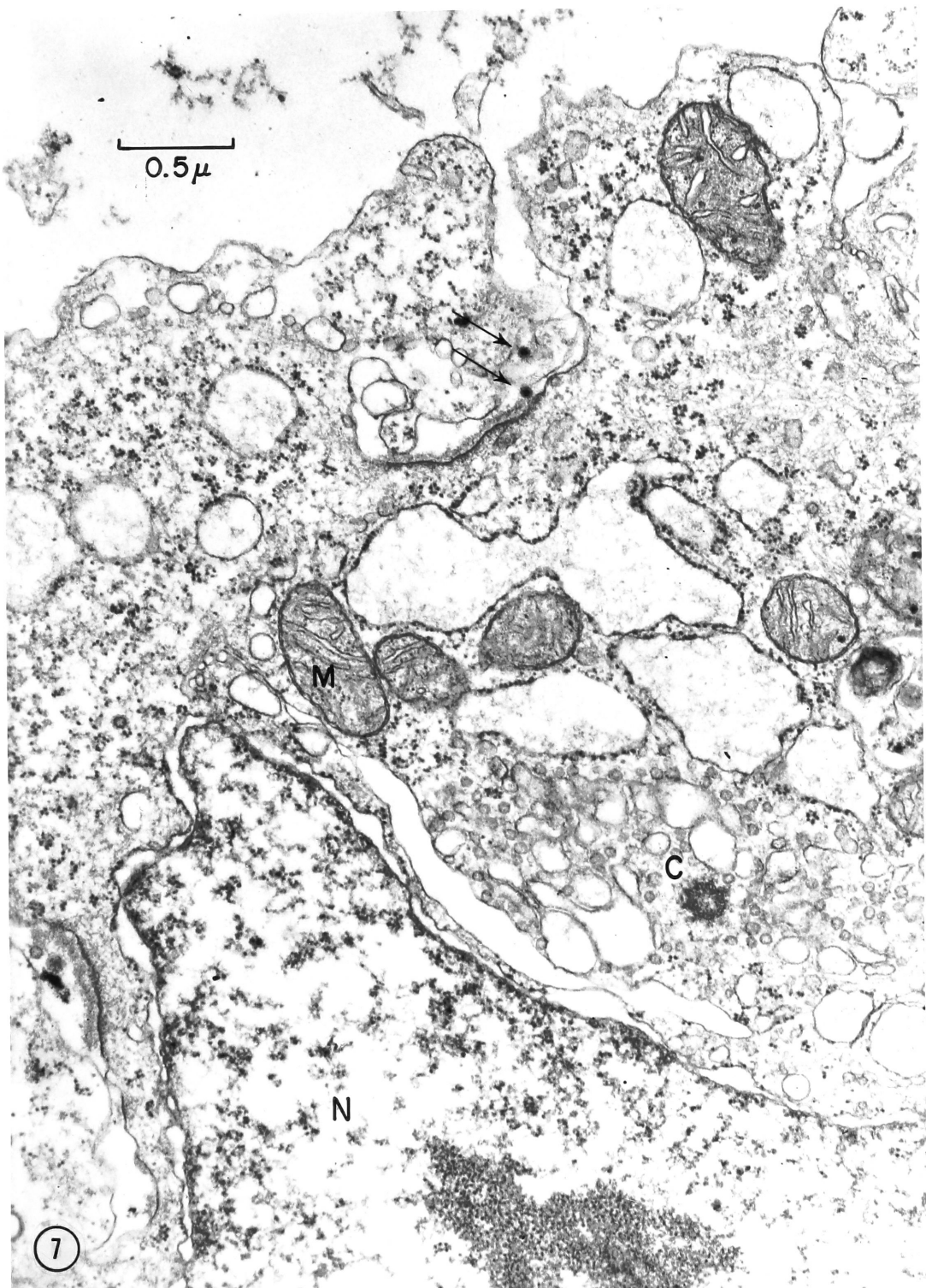
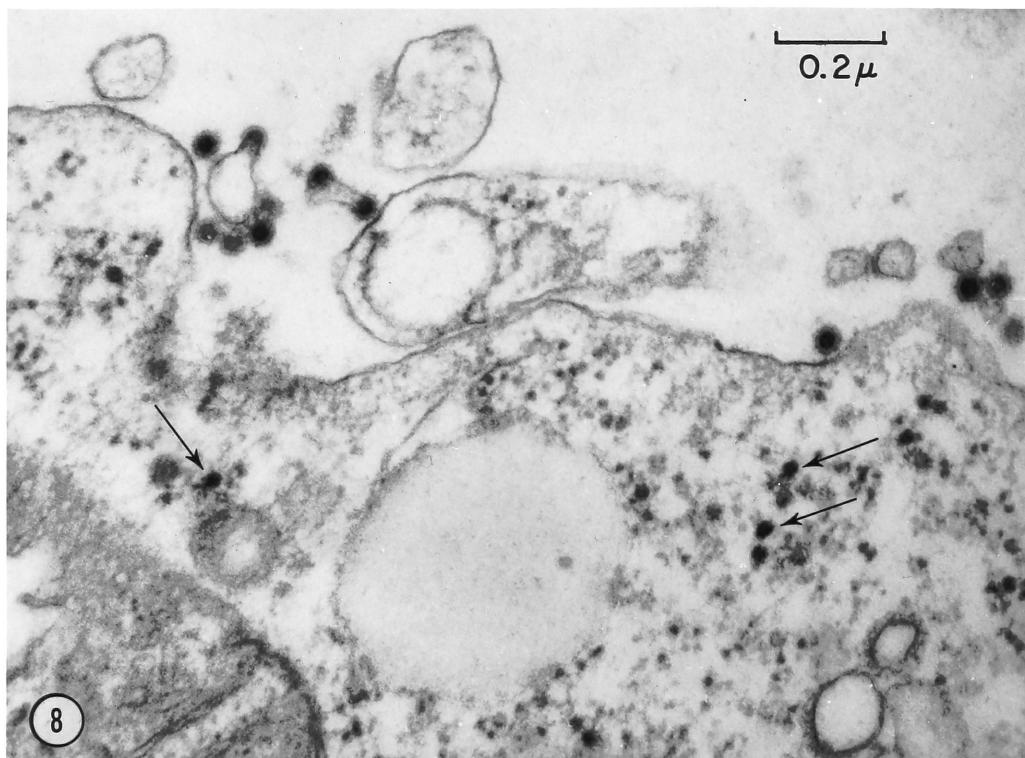


Fig. 8. A portion of a cell sampled 9 hours after infection. Virus particles are budding from cytoplasmic extensions into the extracellular space, and single nucleoids (arrows) are scattered in the cytoplasmic matrix. Magnification: x 68,000.

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a. Structure and site of assembly of nucleoids. Free nucleoids located in the cytoplasmic matrix (Fig. 9) measure on the average 280 A in diameter and have a structure identical to that of the nucleoids of sectioned virus particles. The outer dense shell appears circular in profile. The interior of the nucleoid is less dense, and a dark central spot, which measures approximately 50 A, is often present.

Free nucleoids are seen only in the cytoplasmic matrix of infected cells. No unique site where nucleoids might be assembled within the cytoplasmic matrix has been found. In cells examined 5 hours after infection, nucleoids are not seen preferentially associated either with membranes or with other cytoplasmic constituents.

b. Structure and localization of developing virus particles. Virus particles budding from the limiting membrane of cytoplasmic extensions are shown in cross section in Figs. 10 and 11. The plasma membrane appears to be wrapped closely about the dense nucleoid which is being extruded. In Fig. 10, both leaflets of the unit membrane are made visible by the uranyl acetate stain. It is evident that the triple-layered structure of the unit membrane, present in the plasma membrane of the cell, extends around the developing virus particle. In Fig. 11, only the inner leaflet of the unit membrane can be clearly seen; this sample was not stained in the pellet with uranyl acetate. The location of the outer leaflet of the unit membrane is suggested by a semicircle of density which may mark the site of insertion of the projections in the membrane surface.

Developing virus particles are seen at the limiting membrane of the cell (Figs. 8, 12), or at the membranes of certain intracellular vacuoles (Figs. 12, 13). These vacuoles are usually near the periphery of the cell, and are oval or round in cross-section, with electron-lucent contents. The membranes of these vacuoles do not have attached ribosomes. Virus has never been seen developing at the surface of rough endoplasmic reticulum; it has occasionally been seen within cisternae of the Golgi apparatus (Fig. 19).

Completed virus particles always lie either in the extracellular space or within the lumina of cytoplasmic vacuoles (Figs. 12, 13). Most

Fig. 9. Virus nucleoids in the cytoplasmic matrix. Two of the four nucleoids (arrows) show a characteristic lighter interior and a dark central spot. Magnification: x 135,000.

Fig. 10. Virus particle budding from a cytoplasmic extension, from a sample stained in the pellet with uranyl acetate before sectioning. The triple-layered unit membrane, clearly visible at the cell border, can be followed (arrows) into the developing virus particle. Magnification: x 215,000.

Fig. 11. Virus particle budding from a cytoplasmic extension. The sample was not stained with uranyl acetate in the pellet. The visible part of the cell membrane (arrows), probably representing only the inner leaflet, is wrapped closely about the nucleoid. The outer leaflet may be indicated by the lightly-staining border which forms a semicircle around the developing virus particle. Magnification: x 215,000.

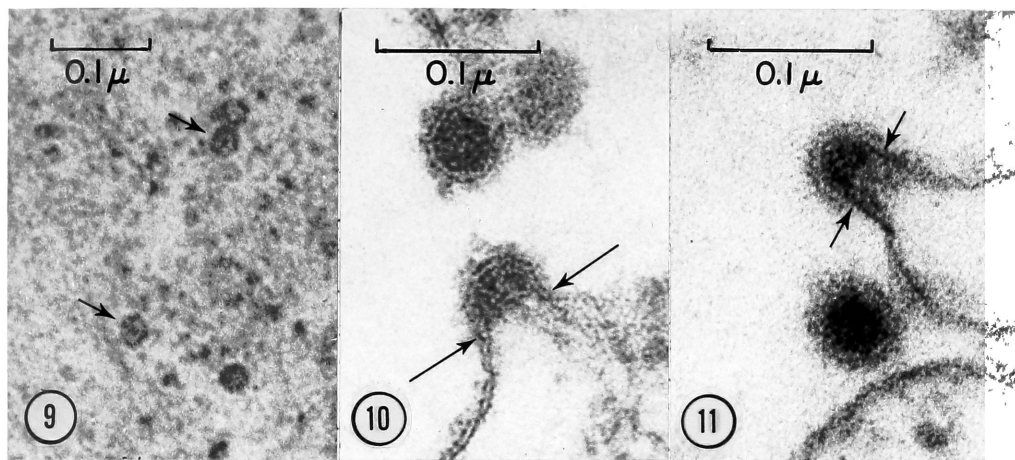
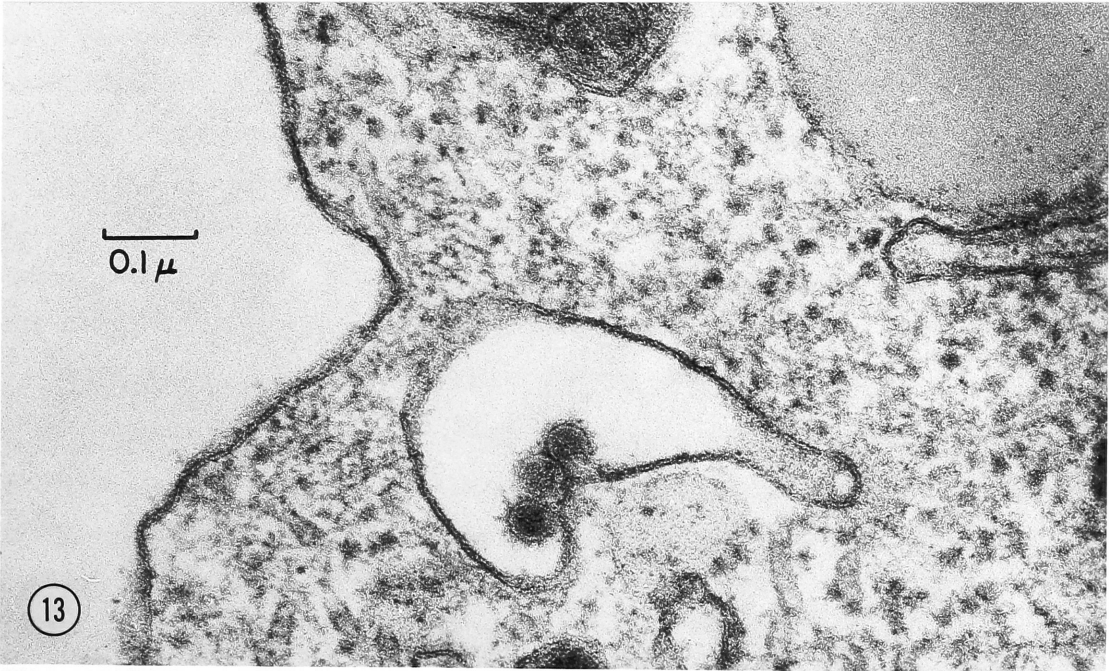
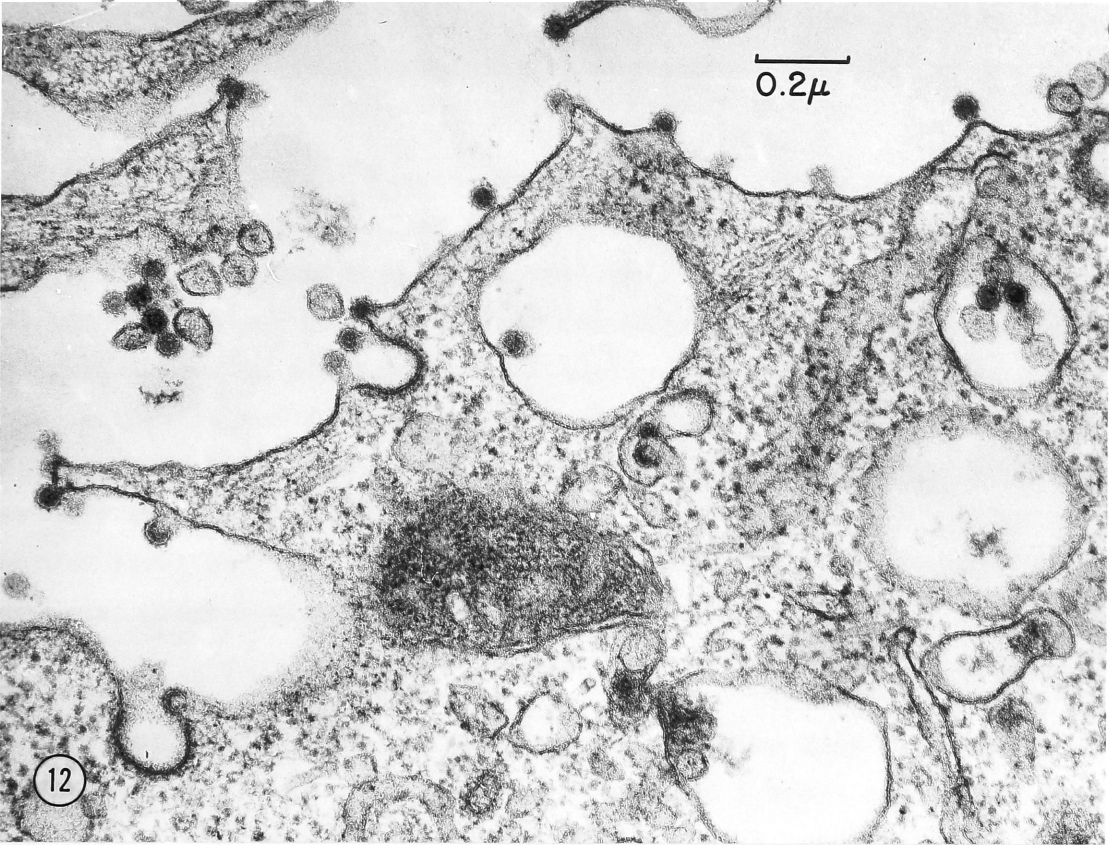


Fig. 12. Developing and complete virus particles at the plasma membrane and within vacuoles near the cell surface. The triple-layered unit membrane is continuous with the envelopes of several developing virus particles. (7 hours; trypsin-released cell; stained with uranyl acetate.) Magnification: x 61,000.

Fig. 13. Three virus particles budding into a small vacuole near the cell surface. (7 hours; trypsin-released cell; stained with uranyl acetate.) Magnification: x 118,000.

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developing and completed particles are located at the plasma membrane; fewer are found within vacuoles. It thus appears that assembly of virus particles takes place predominantly, but not exclusively, at the plasma membrane.

c. Outline of Semliki Forest virus formation. These observations suggest the following scheme of Semliki Forest virus formation. Virus nucleoids are assembled in the cytoplasmic matrix, and migrate singly to the cell border or to certain intracellular membranes. Where a nucleoid comes in contact with the appropriate membrane, a coating of projections is added to the surface of the membrane opposite to where the nucleoid is located. The membrane with the projections is wrapped closely about the nucleoid as it emerges from the surface to form a mature virus particle. The process of virus particle assembly is depicted diagrammatically in Fig. 14.

4. Virus-specific Vesicles

Small membranous vesicles, ranging in size from 350 to 1000 A, are present in large numbers in cells sampled 3 hours after infection and later. The vesicles are bounded by a single unit membrane, and have an irregular dark central spot in an otherwise lightly-staining interior. They are found in two distinct locations: at the outer surface of the plasma membrane (Fig. 15) or lining the inner surfaces of large, round vacuoles in the cytoplasm (Figs. 16, 18). In both places, the vesicles appear to remain attached to the adjoining membrane, for almost all vesicles are located very close to a membrane surface. In some cases the membrane of the vesicle is clearly continuous with the plasma or vacuolar membrane (Figs. 15, 17).

The round vacuoles sometimes contain myelin figures or amorphous material. They are at times surrounded by one or several layers of virus nucleoids (Fig. 18). Sometimes a nucleoid lies in the cytoplasmic matrix directly opposite one of the vesicles (Fig. 16, arrows). Occasionally, virus particles and vesicles can be seen adjacent to each other at a membrane surface (Fig. 19). Such views suggest that these vesicles are produced by a mechanism similar to that by which viral envelopes are formed. The nature of the vesicles, and the role they play in virus development, is unknown.

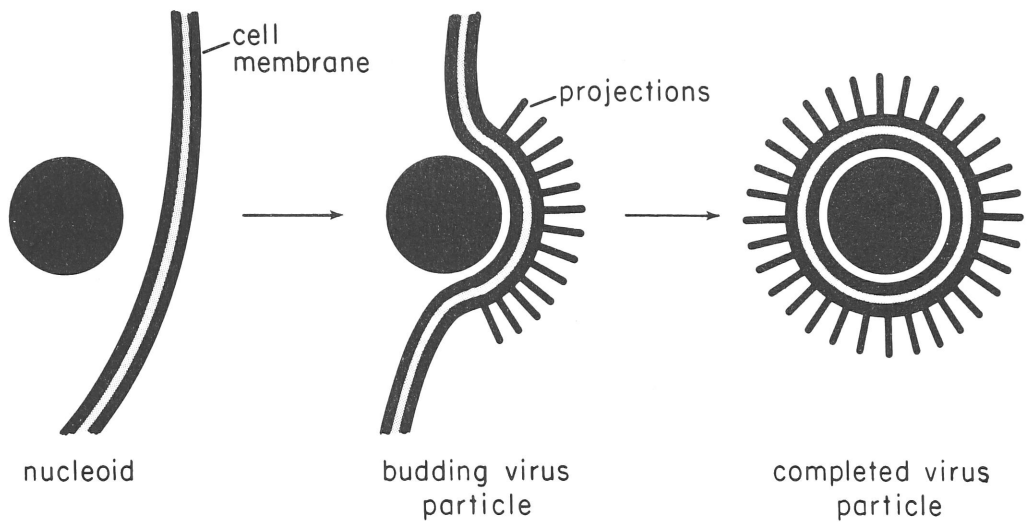


Fig. 14. A schematic diagram illustrating the process of formation of the Semliki Forest virus particle. Left: a virus nucleoid, assembled elsewhere in the cytoplasmic matrix, approaches a cell membrane, represented as a triple-layered unit membrane. Center: as the nucleoid buds through the membrane, a coating of projections is added to the surface of the membrane opposite to where the nucleoid is located. Right: the virus particle, consisting of a nucleoid wrapped in a piece of membrane surrounded by projections, has been completed and lies free in the extracellular space or within a vacuole. The diagram is drawn roughly to scale: the nucleoid is 280 Å in diameter, the unit membrane is 75 Å thick and separated from the nucleoid by a narrow (10–30 Å) space, and the projections are 110 Å long, making the whole virus particle approximately 700 Å in diameter. For the sake of clarity, the projections have been represented as more uniform than they appear in photomicrographs.

Fig. 15. Vesicles budding from the plasma membrane of an infected cell. The unit membrane structure can be seen both in the plasma membrane and in the vesicles. In some locations (arrows) the membrane of a vesicle is clearly continuous with the plasma membrane. (7 hours; trypsin-released cell; stained with uranyl acetate.)
Magnification: x 117,000.

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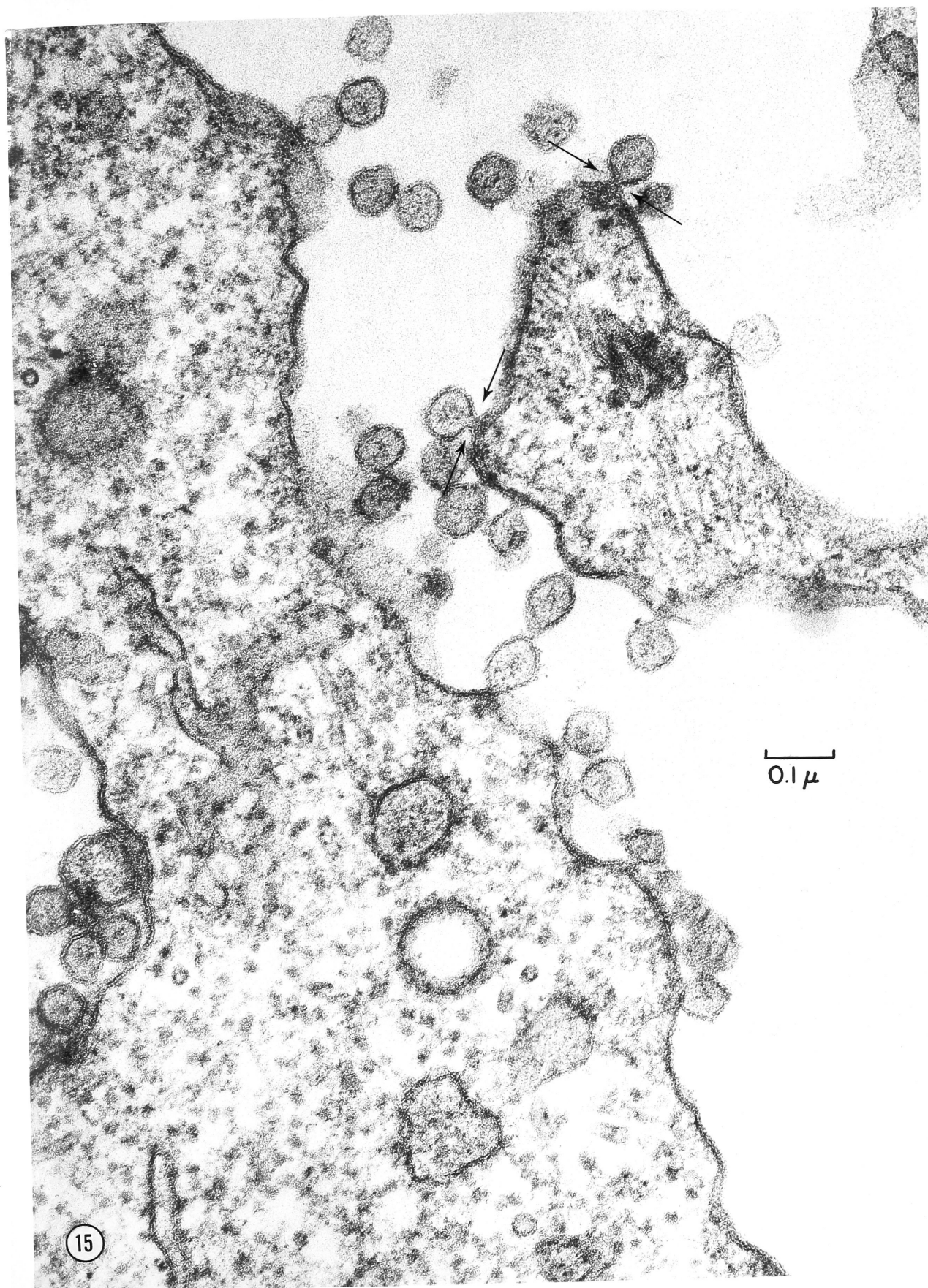


Fig. 16. Two vacuoles lined on the inside of the vacuolar membrane with small vesicles, in the cytoplasm of an infected cell. Several of the vesicles, which contain a dark central area, lie opposite nucleoids in the cytoplasmic matrix (arrows). Dense material within the vacuoles may represent phagocytized substances. (13 hours)

Magnification: x 62,000.

Fig. 17. Two vesicles budding into a vacuole. The unit membrane of the vesicle is continuous (arrows) with the membrane of the surrounding vacuole. Three nucleoids lie in the cytoplasmic matrix in the lower right corner. (13 hours; stained with uranyl acetate.) Magnification: x 130,000.

Fig. 18. A vacuole lined on its inner edges with vesicles, as in Fig. 16, but surrounded by several layers of nucleoids. (13 hours) Magnification: x 92,000.

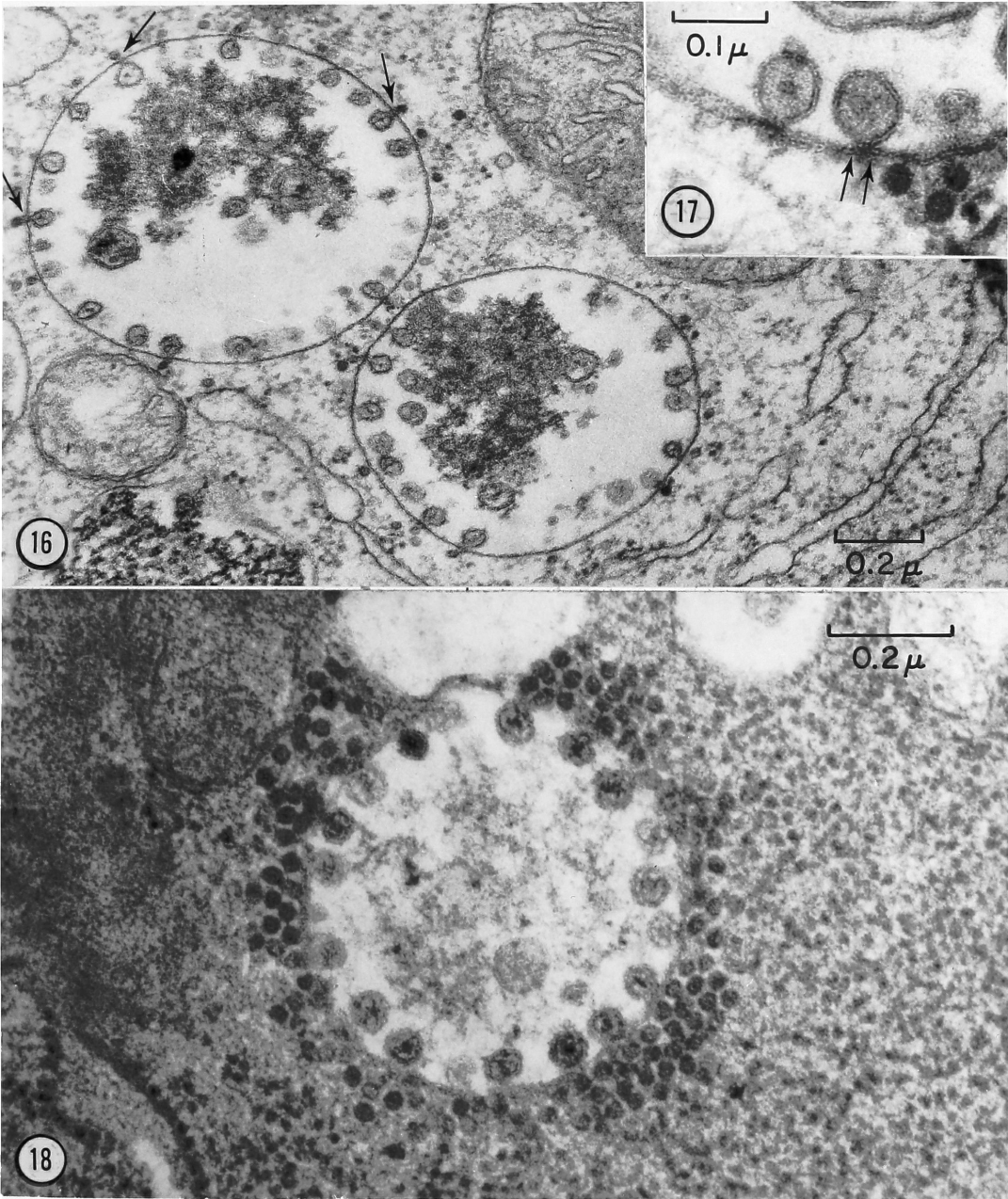
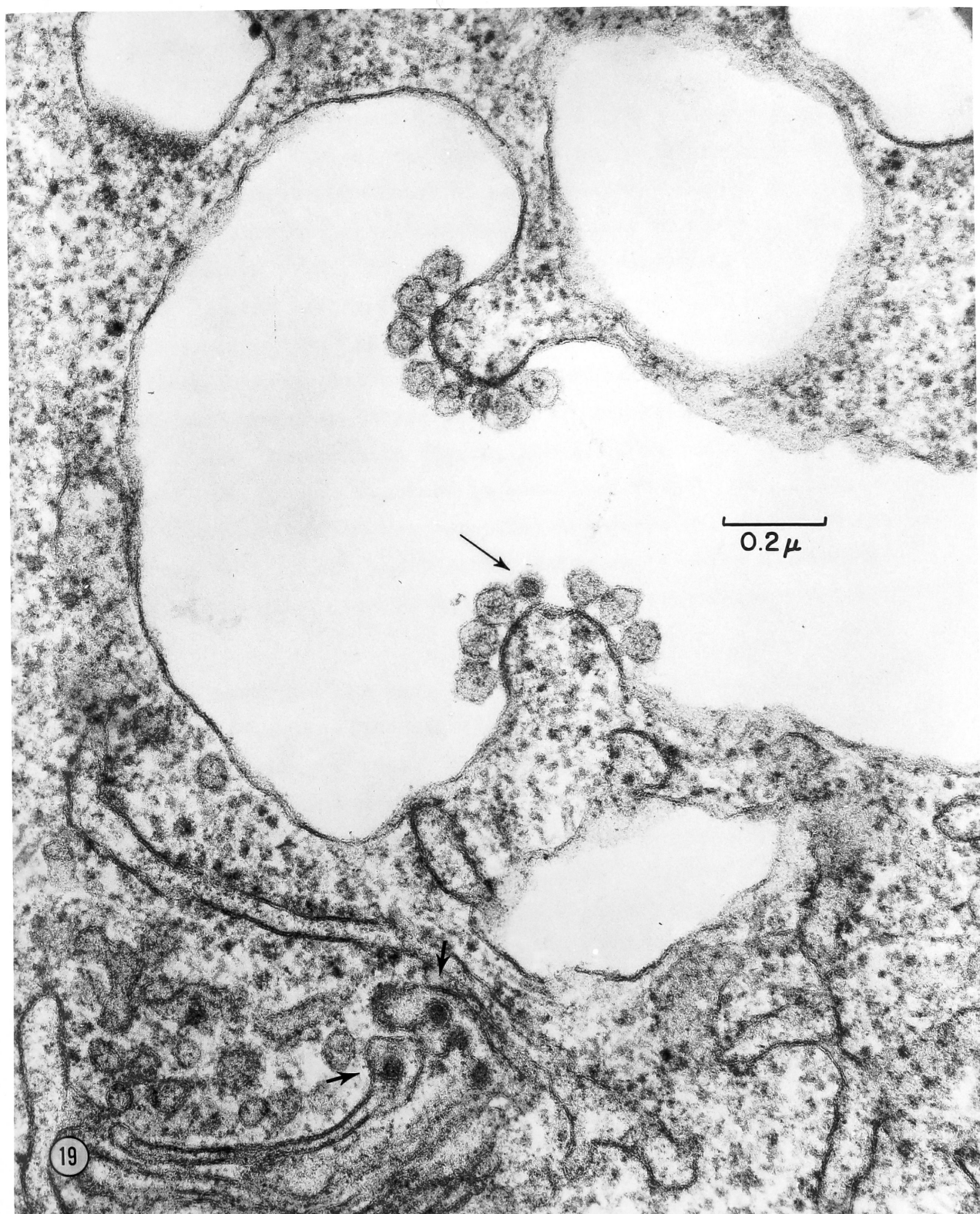


Fig. 19. Vesicles budding into an irregularly-shaped vacuole in the cytoplasm of an infected cell. A single virus particle (thin arrow) which lies among a group of vesicles also appears to have been recently formed at the same membrane surface. In this figure several budding virus particles (thick arrows) can also be seen within the dilated termini of cisternae of the Golgi apparatus. (7 hours; trypsin-released cells; stained with uranyl acetate.) Magnification: x 88,000.



5. Late Events in the Infected Cell

As was described above, a majority of cells exhibit only minor changes in morphology from 5 to 9 hours after infection. These cells are, however, producing virus at the maximal rate. From 9 hours onward, virus production is slower, and eventually ceases 11 to 12 hours after infection. An increasing number of cells become shrunken and rounded, with very densely-staining cytoplasm due in part to massive concentrations of ribosomes (Figs. 20, 21). These concentrations of ribosomes may reflect reduced cell volume. Planar sheets of regularly spaced ribosome tetramers (Fig. 22) are occasionally seen in these shrunken cells. These probably arise by crystallization of the abundant single ribosomes, as appears to happen in cells of intact chick embryos incubated at low temperatures (Byers, 1967). Some cells are highly vacuolated. The nuclear envelope is sometimes absent; dark circular areas of fibrillar texture may represent condensed chromatin. Mitochondria are rare. Rough endoplasmic reticulum is often confined to the edges of the cell where several layers of parallel cisternae may be present.

Virus particles are only rarely seen budding from these condensed, rounded cells, although virus nucleoids are present in great numbers in many cell profiles. Besides being singly dispersed in the cytoplasmic matrix, nucleoids occur in two distinct types of aggregate in these cells: studding the cytoplasmic side of vacuoles, and in paracrystalline arrays. These striking accumulations of nucleoids, which are rarely seen in the early samples, are a characteristic feature of chick embryo cells during terminal stages of the growth cycle of Semliki Forest virus.

a. Nucleoids surrounding vacuoles. Tubular or round membrane-limited vacuoles are seen surrounded by a single layer of nucleoids apposed to the cytoplasmic side of the vacuolar membrane (Figs. 23 to 28). The vacuoles often appear in groups (Figs. 23, 24), especially at later times. They are regularly seen in the vicinity of the Golgi apparatus (Figs. 25), but are found elsewhere in the cell as well. The vacuoles frequently contain hollow cylindrical structures (Figs. 23 to 28)

Fig. 20. A portion of a cell sample 13 hours after infection. The cell is contracted and highly vacuolated. There are many ribosomes, both free and arranged along the borders of parallel cisternae of the endoplasmic reticulum. The cytoplasmic matrix is electron-dense. A single virus particle is seen budding at the cell border at the left-hand side. Virus nucleoids lie at the border of several vacuoles (arrows), which contain vesicles of varying size. The nucleus is absent from this section. Magnification: x 23,000.

Fig. 21. A portion of the cytoplasm of a cell sampled 13 hours after infection. A dark spherical mass, perhaps condensed chromatin, is surrounded by large numbers of ribosomes. Above this mass and in the lower right corner are several collections of nucleoids surrounding vacuoles. Magnification: x 44,000.

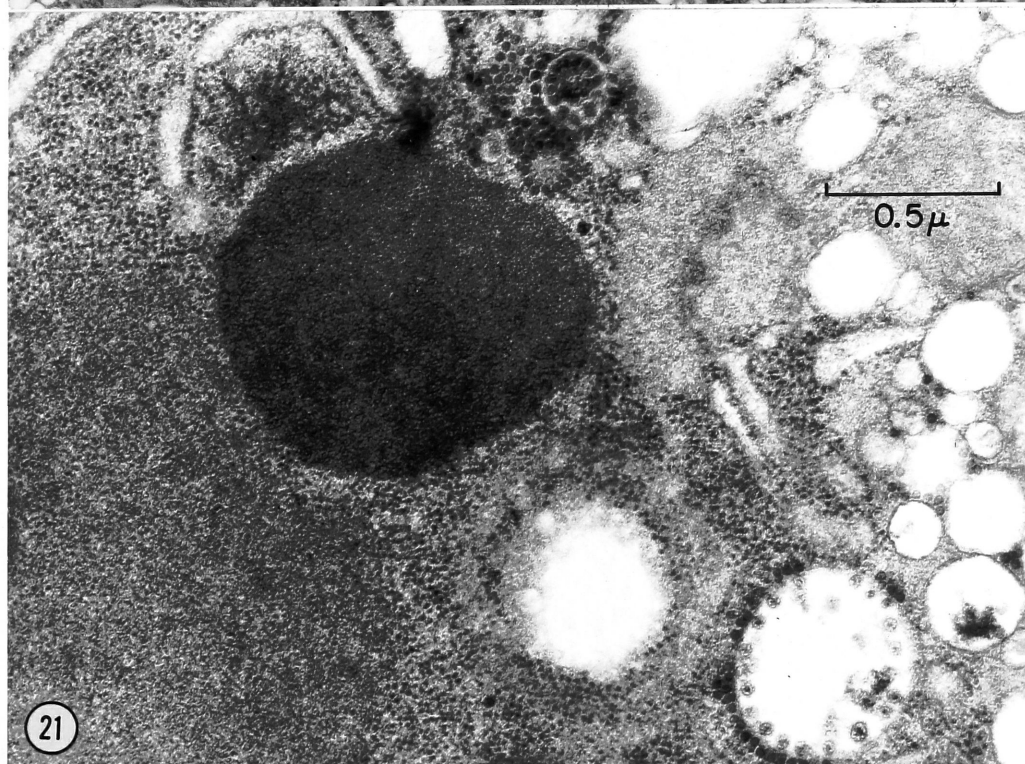
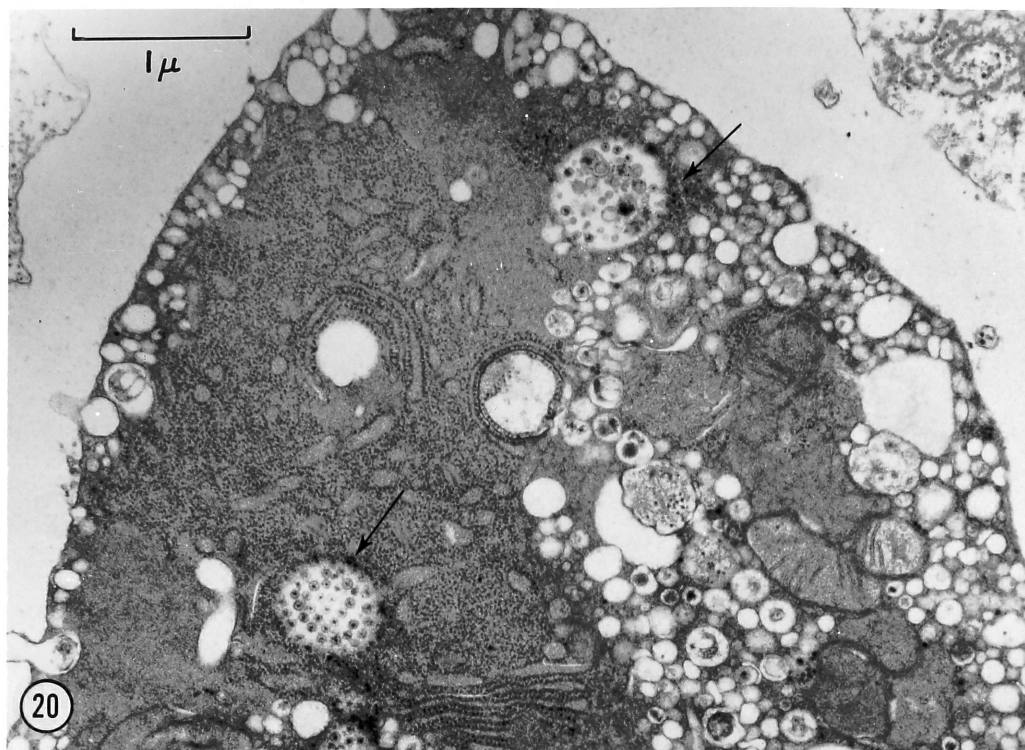


Fig. 22. A portion of a planar array (arrows) of ribosome tetramers present in the cytoplasmic matrix of an infected cell. The pattern can be thought of as a square array of ribosomes with every fifth unit missing (Byers, 1967). It is surrounded by numerous single ribosomes. Some virus nucleoids are seen towards the periphery of the cell. (16 hours) Magnification: x 101,000.

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Fig. 23. A collection of round vacuoles surrounded by nucleoids, in the cytoplasm of a cell sampled 11 hours after infection. Three central vacuoles contain cylindrical structures. Some vacuoles contain virus particles (an infrequent finding) and groups of nucleoids surrounded by a membranous structure. The characteristic lightly-staining interior and dense central spot are visible in the nucleoids. Magnification: x 81,000.

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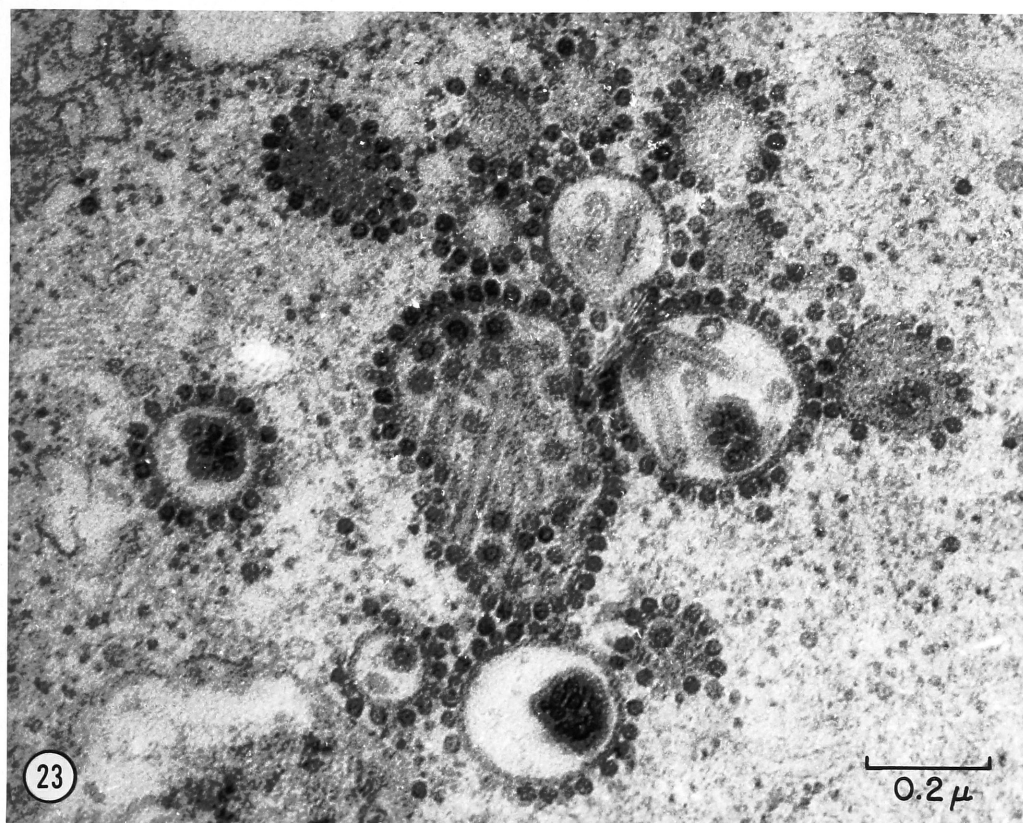


Fig. 24. A collection of tubular vacuoles surrounded by nucleoids.
All of the vacuoles contain cylindrical structures.
(11 hours; trypsin-released cells; stained with uranyl
acetate.) Magnification: x 71,000.

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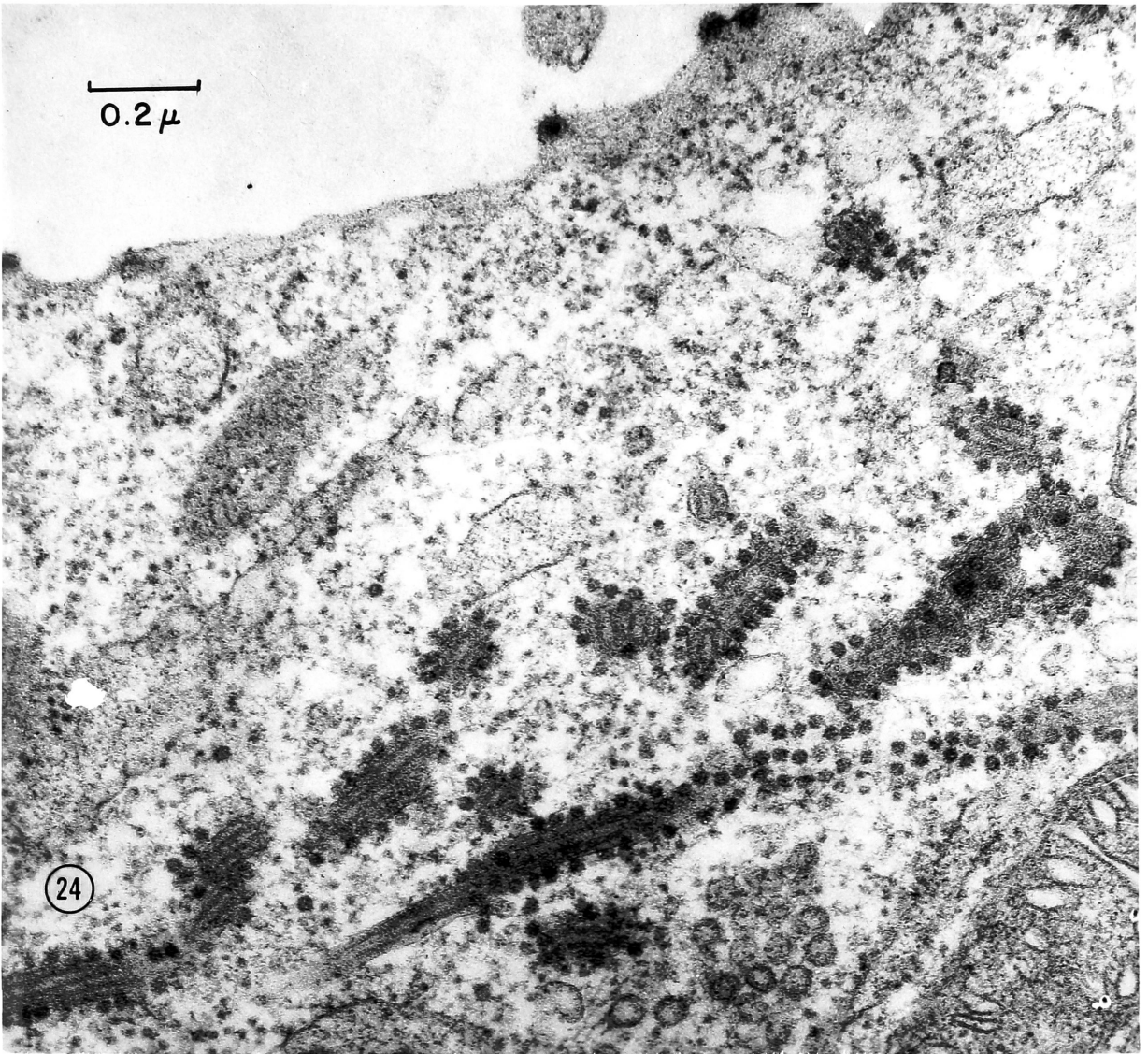


Fig. 25. Numerous small apparently tubular vacuoles (arrows) containing cylindrical structures, in the vicinity of the Golgi apparatus. Several of the vacuoles are lined with nucleoids. Both cisternae and vesicles of the Golgi apparatus are numerous. (7 hours; trypsin-released cell; stained with uranyl acetate.)
Magnification: x 71,000.

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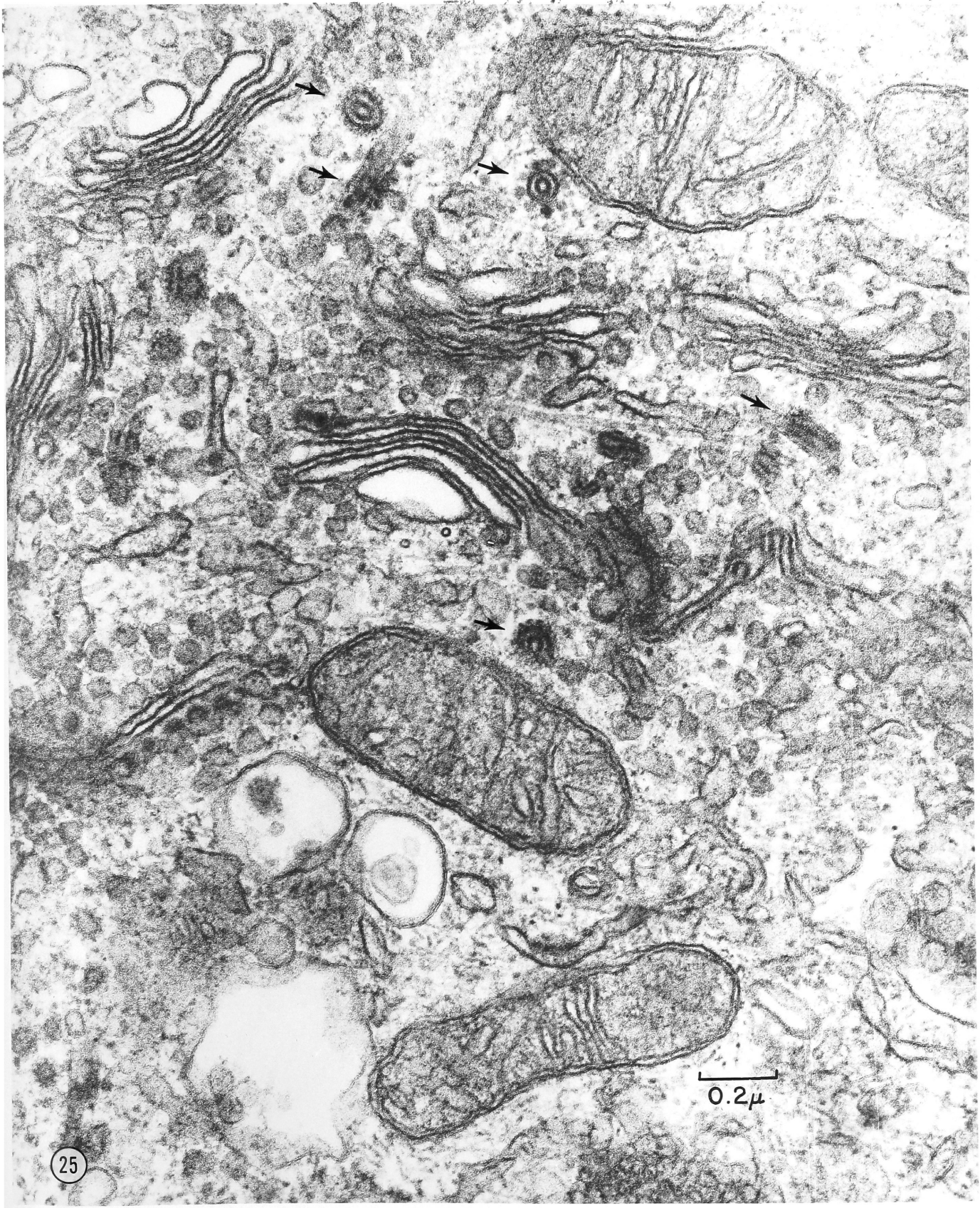
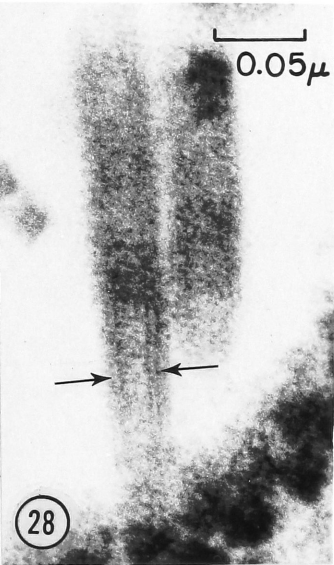
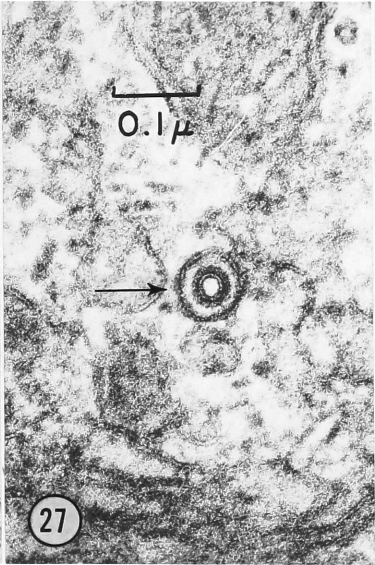
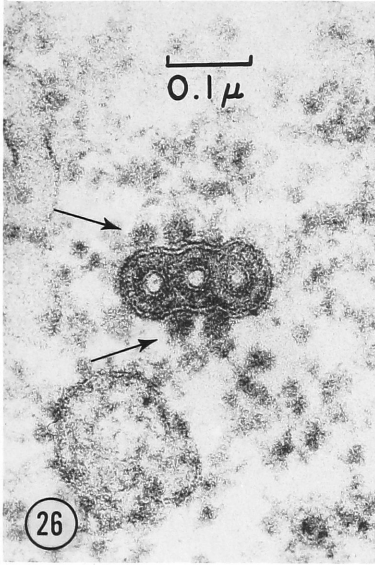


Fig. 26. A cross section of an apparently tubular vacuole which contains three separate cylindrical structures of similar diameter, and is lined on its outer surface with nucleoids. The unit membrane structure of the tubular membrane, and that of the enclosed cylinders, is apparent. (7 hours; trypsin-released cell; stained with uranyl acetate.) Magnification: x 114,000.

Fig. 27. A cross section of an apparently tubular vacuole in which the structure of the enclosed cylinder is especially clear. In the cross section the structure of the cylinder is resolved into a unit membrane, which is surrounded by dense, fuzzy material. The unit structure of the surrounding tubular membrane is also evident. This section of the vacuole contains no attached nucleoids. (7 hours; trypsin-released cell; stained with uranyl acetate.) Magnification: x 110,000.

Fig. 28. Longitudinal sections of two cylindrical structures within the lumen of a cytoplasmic vacuole which is surrounded by nucleoids. The three-layered unit membrane can be seen along both sides (arrows) of one of the cylinders. (13 hours; stained with uranyl acetate.) Magnification: x 245,000.



approximately 400 Å in diameter and up to 1 μ in length. Occasionally, identical tubular vacuoles enclosing cylindrical structures are seen with no attached nucleoids (Fig. 27). In samples which have been stained in the pellet with uranyl acetate, the inner surface of the enclosed cylinder is seen to resemble the three-layered unit membrane structure (Figs. 26, 27, 28). In these views, the outer surface of the enclosed cylinder appears as a fuzzy coat surrounding the inner unit membrane. In some instances the membrane of such a cylinder appears to be continuous with the membrane of the surrounding vacuole (Figs. 23, 24, 25). These views suggest that the cylinders may result from the invagination of a fingerlike projection of the vacuole membrane into the luminal space. Occasionally virus particles are found within vacuoles surrounded by nucleoids (Fig. 23).

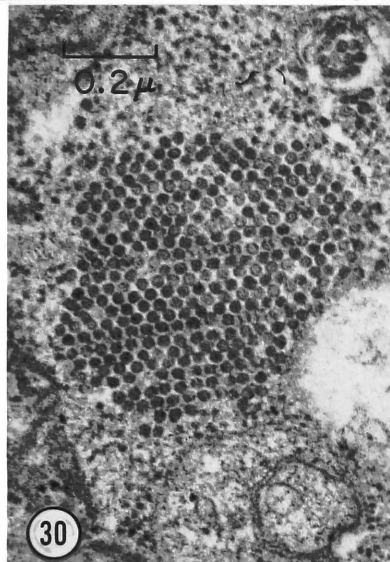
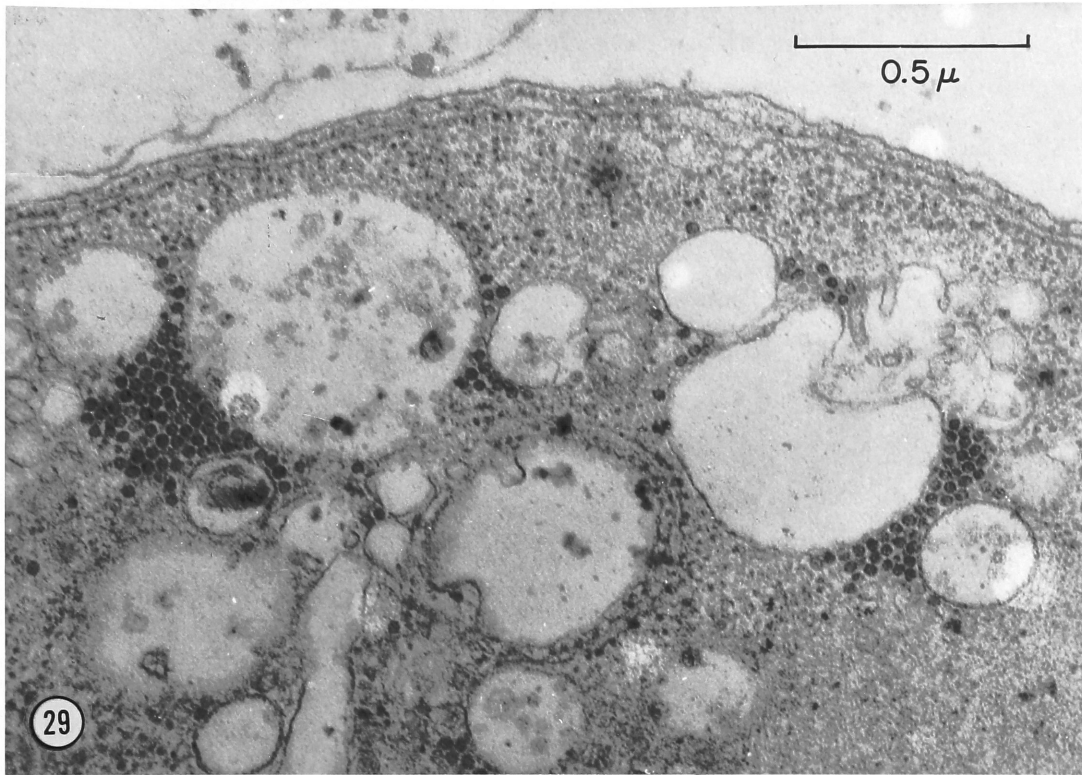
b. Paracrystalline arrays of nucleoids. Nucleoids arrayed in hexagonal or rectangular lattice patterns are first seen in appreciable numbers at 11 hours after infection. Some cells contain numerous small crystalloids of nucleoids packed among vacuoles in the condensed cytoplasmic matrix (Fig. 29). Others contain 1 or 2 large crystalloids (Fig. 30). Crystalloids of over 1000 nucleoids in cross-section have been seen. Such aggregates may contain as many as 30,000 nucleoids.

6. Time Course of Appearance of Virus Particles, Vesicles, and Vacuoles Lined with Nucleoids

To determine the time course of appearance of the various structures seen in infected cells, 90 different cell profiles were examined at each 2-hour interval from 3 to 11 hours after infection. Trypsin-released cells were utilized in order to be able to examine individual cell profiles. Each profile was scored for the presence or absence of (1) developing or complete virus particles, (2) vesicles, either at the plasma membrane or vacuolar membranes, and (3) vacuoles surrounded by nucleoids. Sections were examined at 20,000 magnifications, and orientation within a section was observed at 2,000 magnifications so that no cell profile was counted more than once. When not all 90 cell profiles for a given time were within one section, at least 100 thick (~ 1000 Å) sections were cut before another section was viewed, to avoid bias in the results.

Fig. 29. Nucleoids forming small crystalloids at the edges of vacuoles in a cell sampled 11 hours after infection. Magnification: x 60,000.

Fig. 30. A large crystalloid of nucleoids in the cytoplasmic matrix of a cell sampled 13 hours after infection. Magnification: x 60,000.



The results of the scoring are shown in Fig. 31. The ordinate is a normalized value which represents the number of cell profiles containing the given structure as a percent of the maximum number of profiles containing that structure.

The number of cells containing images of virus particles is low at 3 hours, but nearly reaches a maximum by 5 hours after infection. This correlates well with the growth curve; virus production is just beginning 3 hours after infection, and reaches its maximum rate by 5 hours.

The number of profiles containing vesicles is substantial at 3 hours, and also nearly reaches a maximum by 5 hours. This number remains high through the 11th hour after infection.

In contrast, the number of profiles which contain round or tubular vacuoles lined with nucleocapsids does not reach a maximum until late in the infectious cycle. At three hours, none of the 90 cell profiles viewed contains such a vacuole lined with nucleocapsids. The relative frequency of occurrence of these vacuoles increases with time and reaches a maximum at 11 hours, when 19 of the 90 profiles examined contain vacuoles lined with nucleocapsids. Thus, although virus particles and vesicles appear early in the growth cycle of Semliki Forest virus, vacuoles lined with nucleocapsids are not present in large numbers until late in the cycle.*

* It should be noted that, even if only 1 or 2 percent of cell profiles contain images of the smaller vacuoles, on the average each whole cell might contain at least one vacuole. If we assume the average cell diameter to be 20 μ , and the average thickness of the light gold sections used for counting to be 0.1 μ , then there should be 200 sections per cell. If the length of a typical small tubular vacuole is 0.3 μ , and if its long axis were perpendicular to the plane of sectioning, then such a tube would appear in 3 or at most 4 sections. If there were one 0.3 μ long tube in each cell, only 4/200 or 2% of all sections would contain an image of the tube. This is a maximum value, for the long axis of the tube need not be perpendicular to the plane of sectioning, in which case the tube would appear in fewer sections.

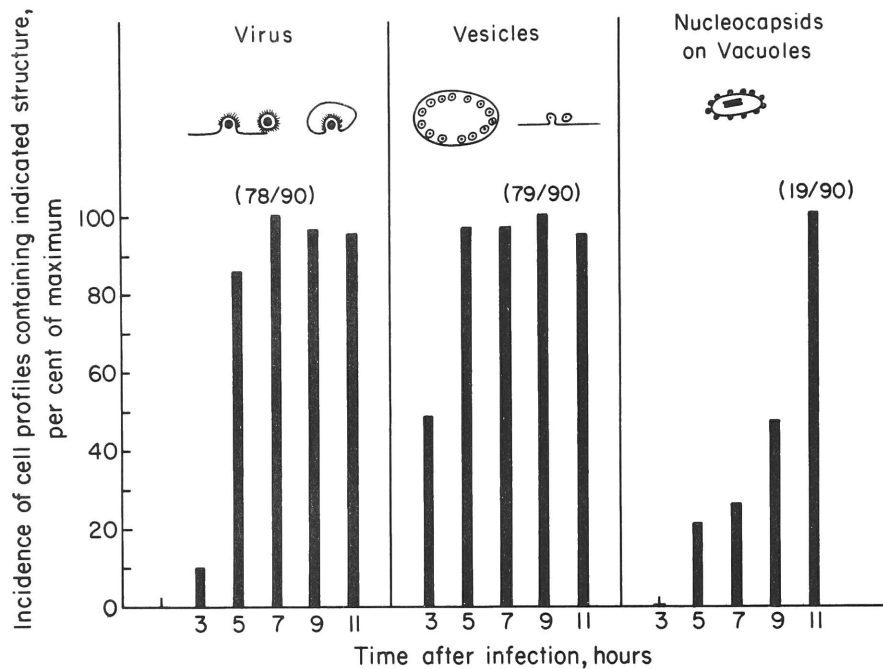


Fig. 31. Time course of appearance of virus particles, vesicles, and vacuoles lined with nucleocapsids. Each bar represents the proportion of cell profiles which contain the indicated structure, expressed as a percent of the maximum number of profiles containing the structure. In each section, the maximum number of such profiles is indicated as a proportion of the total number of profiles (90) examined at each time.

D. Discussion

1. Structure and Assembly of Virus Nucleoids

Semliki Forest virus particles are formed from two parts: a small, densely-staining, spherical nucleoid, and an envelope which is an altered portion of a cellular membrane. The nucleoid, here identified only by its morphological appearance, is presumably the ribonucleo-protein internal component, or nucleocapsid, of the virus; that is, it probably contains the viral RNA and capsid protein. These assumptions are verified in part III of this thesis, in which the purification and analysis of viral nucleocapsids is described. The uniform size and spherical appearance of the nucleoid, and the formation of large regular crystalloids of nucleoids in infected cells, suggest that the nucleoid consists of a symmetrical, closed shell of protein subunits surrounding the viral RNA, analogous to other small, RNA-containing viruses such as poliovirus or turnip yellow mosaic virus (Finch and Klug, 1959; Klug and Caspar, 1960; Finch and Klug, 1966). This subject is more fully discussed in part III of this thesis.

The assembly of nucleoids probably takes place in the cytoplasmic matrix of infected cells, for they appear exclusively within that compartment, either free or adjacent to membranous structures. The exact site of nucleoid assembly is not known. In a single-cycle infection of chick embryo cells with Semliki Forest virus, individual nucleoids scattered in the cytoplasmic matrix are visible when the rate of virus production reaches its maximum, and thereafter. Nucleoids appear to aggregate at the surface of round or tubular membranes, and in paracrystalline arrays. However, these aggregates do not appear in large numbers until late in the course of production of infectious virus. Therefore, it is unlikely that these aggregates are directly involved in virus formation.

Since the publication of most of the present work (Acheson and Tamm, 1967), several electron microscopic studies of cells infected with Semliki Forest virus or the closely related chikungunya virus have appeared (Erlandson et al., 1967; Higashi et al., 1967; McGee-Russell and Gosztonyi, 1967; Grimley et al., 1968). In each case,

nucleoids lining cytoplasmic vacuoles were found. Erlandson et al. (1967) also found that these vacuoles increase in number and size late in the virus growth cycle. However, Grimley et al. (1968) reported that vacuoles lined with nucleoids appear as early as 4 hours after infection of chick embryo cells with Semliki Forest virus, under conditions similar to those employed in the present study. No information was given as to how many cell profiles contain images of these vacuoles or whether the number of vacuoles increases at later times. These authors suggested, on the basis of their findings, that nucleoids are assembled at the surface of the vacuolar membrane.

The difference between our findings and those of Grimley et al. may not be very significant. The quantitative studies shown in Fig. 31 demonstrate that by 5 hours after infection a small number of cell profiles do contain vacuoles surrounded by nucleoids. In fact, the few such structures seen at early times are quite small in size, and calculations (see footnote on p. 42) based on section thickness and cell diameter suggest that even if only 1 or 2 percent of cell profiles contain images of these small vacuoles, on the average each whole cell might contain at least one vacuole. The clear results of both our quantitative and qualitative observations are, however, that these vacuoles increase in prominence in the later stages of the virus growth cycle (9-13 hours). At no time up to 9 hours after infection is any significant fraction of nucleoids associated with vacuoles, and this association with membranes increases as the rate of virus production declines. The evidence thus favors the view that the formation of these vacuoles is secondary to the biosynthetic and assembly stages in virus replication.

Nucleoids may build up in number or concentration late in the growth cycle, especially when the rate of virus production declines after 9 hours. This decline is associated with the development of severe cytopathic changes, including vacuolization and condensation of the cytoplasm. Ribosome crystallization is occasionally seen in cells at this stage. The crystallization of nucleoids, and their association with membranous vacuoles, may be induced or accelerated by the cytopathic effects of virus multiplication. Nucleoids may have

an affinity for cellular membranes, as the assembly of complete virus particles takes place at membrane surfaces. However, certain of the membranes at which nucleoids arrive may not be able to be transformed into viral envelopes, and thus no budding of enveloped nucleoids may take place. This may result in accumulations of nucleoids at these sites. The long cylindrical structures regularly seen within vacuoles lined with nucleoids may be invaginations of these vacuolar membranes induced by the presence of nucleoids or other viral products. Their appearance suggests that they consist of a cylinder of membrane coated with a densely-staining matrix, which could be viral envelope protein.

2. Sites of Viral Biosynthesis

As was discussed in the General Introduction to this thesis, there is evidence that arbovirus RNA synthesis takes place in association with membranous structures. It is also probable, but not convincingly demonstrated, that viral protein synthesis takes place on polyribosomes which are associated with membranes. The particular membranes with which viral RNA and protein syntheses are associated have not been identified. Membrane-associated protein synthesis probably takes place on rough endoplasmic reticulum: that is, on cytoplasmic membranes which have attached ribosomes or polyribosomes. Uninfected chick embryo cells, being primarily fibroblasts, already contain a moderately well-developed rough endoplasmic reticulum, and so a unique site of viral protein synthesis might not be expected to appear.

Cellular RNA synthesis, on the other hand, probably does not take place in the cytoplasm of uninfected vertebrate cells, except within certain cytoplasmic organelles (e.g. mitochondria). Friedman and co-workers (Friedman and Berezesky, 1967; Grimley et al., 1968) have suggested that viral RNA synthesis takes place in association with the vacuoles which contain numerous virus-specific vesicles (Figs. 16 and 18). As evidence, these workers point out that (1) such structures appear very early in the infectious cycle; (2) they are found in the large-particle fraction of cell homogenates, where RNA synthesis is localized (Friedman and Berezesky, 1967); and (3)

autoradiography implicates these vacuoles and the plasma membranes as sites of rapid uptake of labeled uridine (Grimley et al., 1968). However, neither the early appearance of these vesicles nor their localization in a crude large particle fraction which contains numerous distinct types of membranes is sufficient evidence to link them with viral RNA synthesis. In addition, the autoradiographic evidence presented by Grimley et al. (1968) is not convincing. The preservation of cellular ultrastructure in their autoradiographs is poor; no quantitative results of grain localization or statistical analysis is presented; nor is it shown that under the exact conditions of labeling and fixation all of the RNA made is still localized at the membranes where it is thought to be synthesized. And although it is claimed that the autoradiographic evidence implicates cytoplasmic vacuoles lined with vesicles as sites of RNA synthesis, the contradictory statement is made that most of the developed silver grains were concentrated near the external cell membrane. Without more evidence, the hypothesis that these vesicles are sites of viral RNA synthesis remains only a speculation.

It seems more likely that these vesicles could be abnormal forms of the virus envelope, arising in a manner analogous to the formation of the envelopes of mature virus particles at membranes. Some virus-synthesized product might stimulate budding of a small portion of the plasma membrane or of vacuolar membranes. This product could be nucleoids, which for some reason are not incorporated into the resulting membrane bleb, or it could be another viral protein. Views such as Fig. 19, in which an apparently complete virus particle lies among several vesicles at the surface of a vacuole, emphasize the similarity of size, location, and process of formation of vesicles and virus envelopes. "Incomplete" virus particles found in certain arbovirus stocks (Stevens and Schlesinger, 1965; Mussgay and Rott, 1964) have a lower specific infectivity, density, and RNA content than complete virus particles. They hemagglutinate and have the appearance of empty virus envelopes with projections (Sindbis virus: Mussgay and Rott, 1964). The appearance of the vesicles seen in the present study is consistent with some of the features expected of such "incomplete" particles, but there is little evidence of surface projections.

The possibility that these vesicles, instead of being produced by an abortive budding process, are phagocytized remains of virus particles is suggested by the resemblance between the vacuoles which often contain them and phagocytic or autolytic vacuoles. However, this would not explain the frequent appearance of the vesicles at the plasma membrane. In addition, it is doubtful that either the input virus or the progeny virus could account for the large numbers of these vesicles seen within each vacuole. Such vesicles have not been seen during the first hour after inoculation of cells with 10 times as much virus as used in the present experiments (Acheson, unpublished results). Thus these vesicles do not appear to be directly related to the uptake of virus by cells.

3. Structure and Assembly of the Virus Particle

In the final step of formation of the Semliki Forest virus particle, the viral nucleoid becomes enveloped in an altered portion of a cellular membrane, most often the plasma membrane. Pfefferkorn and Hunter (1963) showed that the phospholipids of Sindbis virus are derived from pre-formed cellular phospholipids, while Pfefferkorn and Clifford (1964) demonstrated that most of the protein incorporated into virus particles is newly synthesized after infection. Strauss et al. (1968) recently showed that most of the protein in the envelope of Sindbis virus migrates as a single peak in electrophoresis and is thus probably one specific type of polypeptide chain, synthesized under the direction of the virus. Our observations indicate that a portion of the plasma membrane, visible as a three-layered unit membrane, is directly incorporated into the virus particle during virus assembly, as part of the envelope. However, in the process of virus assembly the membrane becomes altered by the addition of projections. If there is only one kind of polypeptide chain present in the envelope, then it probably serves both as a structural protein in the membrane portion of the envelope, and also as the projections. It is not known whether there may also be carbohydrate in the projections.

Lipid analyses of Sindbis (Pfefferkorn and Hunter, 1963) and Semliki Forest (Friedman and Pastan, 1969) viruses show that the distribution of viral phospholipids and cholesterol resembles that of the

plasma membranes of various cell types (Ashworth and Green, 1960; Klenk and Choppin, 1969; Skipski et al., 1965). In the present study, the formation of Semliki Forest virus particles is seen to take place predominantly at the plasma membrane of the cell. However, budding and completed particles are regularly seen at the inner surface of cytoplasmic vacuoles, and occasionally within cisternae of the Golgi apparatus. In many cases these vacuoles may arise by invagination from the plasma membrane. In no case was virus seen budding into cisternae of the rough endoplasmic reticulum.

III. PURIFICATION AND PROPERTIES OF SEMLIKI FOREST VIRUS NUCLEOCAPSIDS

A. Introduction

The electron microscopic study described in part A established that Semliki Forest virus particles consist of two parts: a small, spherical nucleocapsid and an envelope which is an altered portion of a cellular membrane. To understand the assembly and structure of the virus particle further, it is important to determine the structure and properties of the nucleocapsid itself. The size and shape of the nucleocapsid, and the finding of large paracrystalline arrays of nucleocapsids in infected cells, suggested that Semliki Forest virus nucleocapsids have a structure similar to that of the small isometric RNA-containing viruses such as poliovirus or turnip yellow mosaic virus. It was predicted by Crick and Watson (1956) that small, apparently spherical virus capsids are constructed from a number of identical protein subunits arranged symmetrically about the viral nucleic acid to form a closed shell. Caspar and Klug (1962) later showed that the type of symmetry most favored by energy considerations is icosahedral symmetry. They described the allowable numbers and arrangements of subunits which could form closed shells with icosahedral symmetry. All isometric viruses whose structure has been adequately analyzed have been shown to exhibit icosahedral symmetry.

To study the structure and properties of the nucleocapsid of Semliki Forest virus it was necessary to isolate it in pure form, so that chemical and physical measurements could be carried out. Previous attempts to isolate nucleocapsids from intact virus particles by treatment with ether or with detergent had apparently failed (Mussgay and Horzinek, 1966; Osterreith, 1964). Thus it was decided to attempt to isolate the nucleocapsids from infected cells. Electron microscopic observations revealed that late in the infectious cycle there were large numbers of free nucleocapsids in the cytoplasm of infected cells. The nucleocapsids are approximately the size of poliovirus, which has a sedimentation coefficient of 160 S. It was reasoned that Semliki Forest virus nucleocapsids might sediment at a similar rate in sucrose gradients. In fact, Wecker and Richter (1962) had reported that a structure which contains infectious viral RNA and sediments somewhat faster than single ribosomes could be isolated from cells infected

with western equine encephalitis virus, another group A arbovirus. We therefore undertook sucrose gradient analysis on extracts of Semliki Forest virus-infected cells in which structures containing viral RNA had been specifically labeled. The nucleocapsids were located by this means, and further purified. The method of purification will be described, and data reported on the morphology, buoyant density, and ultraviolet absorption spectrum of purified nucleocapsids. The size of the RNA and the number and approximate molecular weights of the proteins from purified nucleocapsids will also be reported. In addition, we have shown that the RNA within nucleocapsids is accessible to digestion by ribonuclease.

B. Materials and Methods

1. Buffers and Chemicals

Buffers were: RSB = 0.01 M Tris-hydroxymethylaminomethane, pH 7.3, 0.01 M NaCl, 0.0015 M $MgCl_2$; PN = 0.01 M sodium phosphate, pH 7.3, 0.1 M NaCl; TEN = 0.01 M Tris-hydroxymethylaminomethane, pH 7.3, 0.1 M NaCl, 0.001 M EDTA.

Sodium dodecyl sulfate (SDS) was recrystallized from ethanol or was 95% grade, Matheson, Coleman and Bell, Cincinnati, O.; sucrose was ribonuclease-free grade from Schwarz BioResearch, Orangeburg, N.Y.; deuterium oxide, 99.9 mole percent, was purchased from Bio-Rad Laboratories, Richmond, California; sodium deoxycholate was Mann-assayed, special enzyme grade, Mann Research Laboratories, New York, N.Y.; pancreatic ribonuclease, crystallized from ethanol, was from Worthington Biochemical Corp., Freehold, N.J.; potassium tartrate, crystal, was from J.T. Baker Chemical Co., Phillipsburg, N.J.; cesium chloride, optical grade, was from Harshaw Chemical Co., Cleveland, O.; Sephadex G-200 from Pharmacia Fine Chemicals, Uppsala, Sweden.

Protein was assayed by the Lowry method (Lowry, 1951); crystalline bovine serum albumin was used as a standard. RNA was assayed by the Orcinol method (Mejbaum, 1939). Orcinol was recrystallized before use. Purified yeast RNA (Sigma type XI) was used as a standard.

The following radioactively labeled compounds were used: L-leucine - 4,5- 3H , 30-50 Ci/mM, New England Nuclear Co.; uridine - 5- 3H , >1000 mCi/mM, Amersham-Searle Corp.; reconstituted protein hydrolyzate- 3H , ~ 1 Ci/mM, Schwarz BioResearch Inc.

2. Purification of Virus

Monolayer cultures of chick embryo cells grown in lactalbumin medium in 100 mm Petri dishes were washed once with PBS and incubated at 37 C in an atmosphere of 5% CO₂ with 10-50 PFU/cell of virus in 1.5 ml reinforced Eagle's medium containing 1% bovine serum albumin. After 1 hour, 3.5 ml reinforced Eagle's medium was added per dish, and incubation was continued for a further 8-11 hours. Cells were harvested by scraping and pelleted. The supernatant fluids, containing the virus, were centrifuged for 10 minutes at 10,000 x g in the Spinco S-30 rotor, and the sediment discarded. Virus was pelleted from the supernatant at 73,000 x g for 1 1/2 hours in the S-30 rotor. The pellets were resuspended in PN buffer containing 5% (w/w) potassium tartrate, and layered over a linear 20-30% (w/w) potassium tartrate gradient in PN. This was spun in the SW-27 rotor at 90,000 x g for 10-15 hours, by which time a single light-scattering band had formed in the middle of the gradient. This band was collected, diluted and rebanded in a second tartrate gradient.

Fig. 32 shows that the profiles of absorbance and radioactivity in ³H-leucine-labeled virus exactly coincide after the second banding in tartrate, and that essentially no contaminating material is present elsewhere in the gradient. Recovery of infectivity was 50-80% of the initial value. The virus band was collected and dialyzed vs PN or RSB prior to analysis of proteins or RNA.

3. Counting of Acid-precipitable Radioactivity

Except as noted, aliquots of 0.05 or 0.1 ml were spotted onto 2.4 cm discs of Whatman No. 3 MM filter paper numbered by pencil and supported with brass pins (Mans and Novelli, 1961). After drying, the discs were fixed in batches in 5% trichloroacetic acid (TCA) for 10 min at 4 C and washed once in 5% TCA. The discs were dehydrated sequentially in 95% ethanol, 100% ethanol and diethyl ether, allowed to dry, and counted in a Packard Model 3375 liquid scintillation counter in 5 ml of toluene containing 160 ml Liquifluor (Pilot Chemicals, Watertown, Mass.) per gallon.

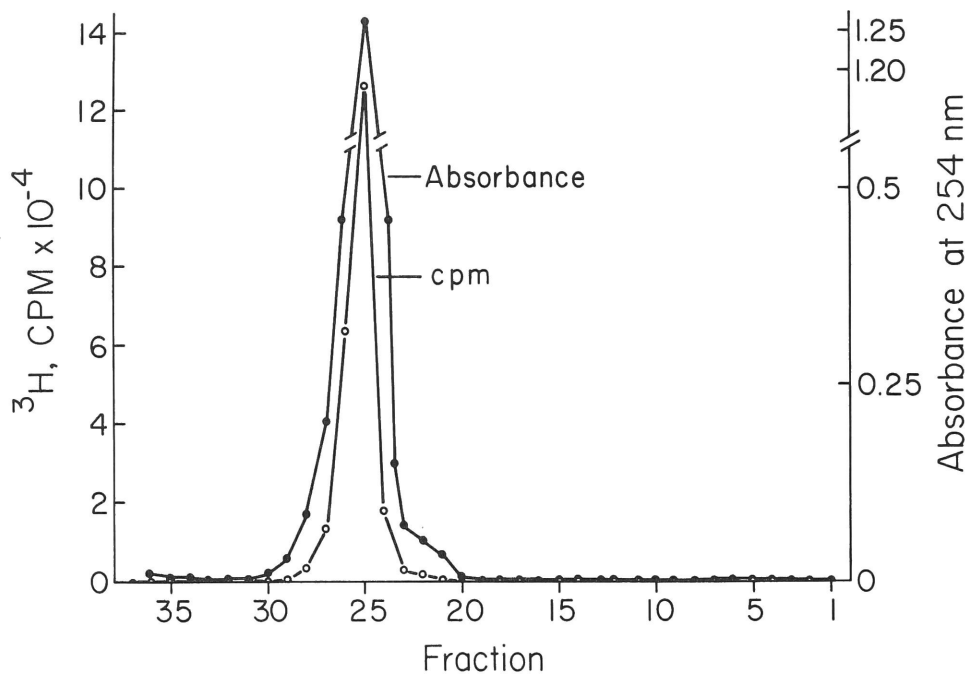


Fig. 32. Purification of Semliki Forest virus on a potassium tartrate density gradient. ^3H -uridine labeled virus was harvested and purified as described in Materials and Methods. The virus band from the first 20-30% (w/w) potassium tartrate gradient was rebanded on an identical second gradient, shown here. Absorbance at 254 nm was continuously monitored as the gradient was collected, and acid-precipitable counts per minute were determined on 0.05 ml aliquots of each 1 ml fraction.

4. Electron Microscopy

Negative staining (Brenner and Horne, 1959) was carried out on copper grids covered with Formvar and a carbon film. A solution of bovine serum albumin, 50 μ g/ml, was applied to wet the grid surface, followed by nucleocapsids in PN buffer and then by 1% potassium phosphotungstate, pH 6.2. Grids were air-dried and examined in a Siemens Elmiskop I electron microscope at magnifications of 20,000 or 40,000.

5. Extraction of RNA

RNA was extracted from nucleocapsids or virus with 1% SDS for 5 minutes at 37 C. Carrier chick embryo RNA was added and the RNA was precipitated by addition of 2.5 volumes of 95% ethanol. After at least 2 hours at -20 C the RNA was pelleted and resuspended in TEN buffer.

6. Sucrose Gradient Analysis of RNA

Samples (0.1 or 0.2 ml) of RNA in TEN buffer were layered over 4.6 ml linear gradients of sucrose, 5-20% (w/w) in TEN, and spun at 4 C in the Spinco SW-39 or SW-50 rotor at 115,000 x g for 2 1/2 hours, or in the SW-50.1 rotor at 230,000 x g for 1 1/4 hours. Fractions of 0.2 ml were collected by displacement with 40% sucrose, and absorbance was monitored at 254 nm with an ISCo flow cell and absorbance analyzer.

7. Polyacrylamide Gel Electrophoresis

7.5% polyacrylamide gels 12 cm in length were polymerized in glass tubes 0.6 cm in diameter. The gels contained 7.5% (w/v) acrylamide, 0.2% N,N'-bis-methylene acrylamide, 0.075% ammonium persulfate, and 0.05% (v/v) N,N,N',N'-tetramethylethylenediamine, in 0.1 M sodium phosphate buffer, pH 7.2, with 0.1% SDS (Summers et al., 1965). A 0.5 cm long spacer gel consisting of 2.5% acrylamide was subsequently formed above each gel. Gels were pre-run for 2 hours at 3 volts/cm to remove unreacted persulfate.

Nucleocapsid or virus samples in PN or RSB were made 1% in SDS and β -mercaptoethanol, and heated in a boiling water bath for 1 minute. One-tenth volume of 60% (w/w) sucrose and of bromphenol blue

(tracking dye) were added, and 25 to 500 μ l samples were layered onto gels. Electrophoresis was carried out at room temperature for 1 hour at 2 volts/cm, to allow the proteins to enter the spacer gel, and then for 12-16 hours at 3 volts/cm.

Gels were extruded from the tubes and either stained or frozen on dry ice for slicing and counting. To stain, gels were fixed for 18 hours in 20% (w/v) sulfosalicylic acid, stained for 18-24 hours in 0.25% Coomassie Blue, and washed in several changes of 7% acetic acid for 24 hours. Frozen gels were sliced into 1 mm segments (Fromageot and Zinder, 1968) which were placed into scintillation vials. Each slice was allowed to swell for 1-2 hours in 0.05 ml water. Then 0.2 ml NCS (Amersham-Searle, Inc.) was added and after 1-2 hours 0.3 ml more NCS was added. Vials were capped and stored overnight and then 10 ml of toluene plus Liquifluor were added. The samples were counted in the Packard scintillation counter.

C. Results

1. Purification of Nucleocapsids

a. Specific labeling of viral RNA. The observation that chick embryo cells infected with Semliki Forest virus contain large numbers of free nucleocapsids at times (11 hours after infection) when virus production has ceased prompted us to attempt isolation of these nucleocapsids for purposes of characterization of their structure and composition. To locate nucleocapsids during cell fractionation procedures, viral RNA was specifically labeled by treating cells with Actinomycin D to inhibit cellular RNA synthesis. The replication of Semliki Forest virus is not inhibited by Actinomycin D in the concentrations used (Taylor, 1965, and Table II). The proportion of newly-synthesized RNA that is viral was determined by comparing the Actinomycin D-resistant RNA synthesis in infected and uninfected cells.

Monolayers of chick embryo cells in 60 mm Petri dishes were incubated with Actinomycin D (2 μ g/ml) or in the absence of the drug, for 2 hours. One treated and one untreated dish were washed with PBS and infected with 40 PFU/ml of Semliki Forest virus in reinforced Eagle's medium containing 1% bovine serum albumin. Two other dishes

were washed and incubated in the same medium but with no virus. After one hour the inoculum was removed, 2 ml of medium containing 2 μ c/ml 3 H-uridine was added to each dish, and cells were incubated for an additional 10 hours. Cells were trypsinized, pelleted, and collected on 0.65 μ pore size Millipore filters, and thoroughly washed with 20 ml cold 5% TCA. The filters containing the acid-insoluble radioactivity were dried and counted in toluene plus Liquifluor in the scintillation counter.

The results of this experiment (Table II) show that Actinomycin D treatment reduces cellular RNA synthesis to 1% of normal, and infection raises this value to 50% of the uninfected untreated control. Thus approximately 98% of the RNA synthesized from 1 to 11 hours after infection of Actinomycin D-treated cells is viral RNA.

b. Extraction of RNA-containing structures from infected cells. Next, experiments were done to determine whether Dounce homogenization efficiently extracts structures containing viral RNA from infected cells. Cells were treated with Actinomycin D, infected, and labeled as before. Twelve hours after infection, the cells were scraped from the surface of the Petri dishes, pelleted, resuspended in cold RSB, and disrupted with 50 strokes of a tight-fitting Dounce homogenizer. Microscopic examination of the homogenate showed that all cells are broken by this treatment, but nuclei remain intact. Nuclei and debris were pelleted at 1000 x g for 15 minutes. The pellet was resuspended in RSB, rehomogenized, and the homogenate spun 1000 x g for 15 minutes. Aliquots of each supernatant and pellet were assayed for acid-precipitable radioactivity.

After vigorous Dounce homogenization of the infected cells, 68% of the label in RNA remained in the clarified supernatant. A second homogenization of the pellet released only an additional 6% of the counts. These results indicate that the homogenization procedure adequately releases structures containing viral RNA into the supernatant, free from nuclei and large debris.

TABLE II

Effect of Actinomycin D on RNA Synthesis and Yield of
Semliki Forest Virus in Chick Embryo Cells

	Actinomycin D ^a	RNA synthesis, ^b % of uninfected control	Virus yield, ^c PFU/ml
Uninfected cells	-	100	-
	+	1.1	-
Infected cells	-	54	7.9×10^8
	+	49	7.5×10^8

^a 2 μ g/ml for 2 hours prior to infection

^b ³H-uridine incorporation from 1 to 11 hours after infection

^c 12 hours after infection

c. Isolation of the nucleocapsids. The clarified supernatant from Dounce homogenization was next analyzed by sedimentation through a 28 ml linear 5-20% (w/w) sucrose gradient made up in RSB. Centrifugation was for 2 hr at 24,000 rpm (55,000 x g) in the Spinco SW-25.1 rotor at 4 C. The gradient was collected by displacement with 40% sucrose pumped into the bottom of the centrifuge tube. Absorbance at 254 nm was monitored with an ISCo flow cell, and 1 ml fractions were collected. Aliquots (0.5 ml) of each fraction were assayed for acid-precipitable radioactivity.

The results of this experiment are shown in Fig. 33. There is a peak of radioactivity which sediments at 145 S, when compared with the single ribosome peak (75 S) revealed by the absorbance tracing. As viral RNA has a sedimentation coefficient of approximately 45 S, and complete virus particles sediment at 300-350 S, it appeared likely that the 145 S peak represents the viral nucleocapsids.

d. Effect of deoxycholate or EDTA on recovery of nucleocapsids. Nearly one-half the labeled RNA was found in the pellet of such a gradient. It was conceivable that many nucleocapsids are bound to, or trapped within, membranous structures which sediment at a high rate. To determine if this is the case, a clarified homogenate of infected, labeled cells was prepared as before; one-half was incubated with 0.5% deoxycholate for 10 minutes at 37 C, while the other half was incubated without detergent. Both were spun on 5-20% sucrose gradients and the fractions were assayed for acid-precipitable radioactivity. The results are shown in Table III. The number of counts in the 145 S region is unaffected by detergent treatment of the homogenate, although four-fifths of the counts in the pellet are released. Most of these counts do not appear elsewhere in the gradient; nucleases which may digest the released RNA are perhaps activated in the detergent-treated cells. It seems clear, however, that deoxycholate treatment does not release an appreciable quantity of nucleocapsids from heavy structures into the 145 S region, nor does it disrupt the 145 S nucleocapsids. Similar results were obtained using 0.25% Triton X-100, a nonionic detergent, in place of deoxycholate.

In the same experiment, the cells were homogenized in a buffer containing EDTA (0.01 M Tris, pH 7.3, 0.001 M EDTA) to determine the effect of removal of Mg^{++} ion on the distribution of label in the gradient.

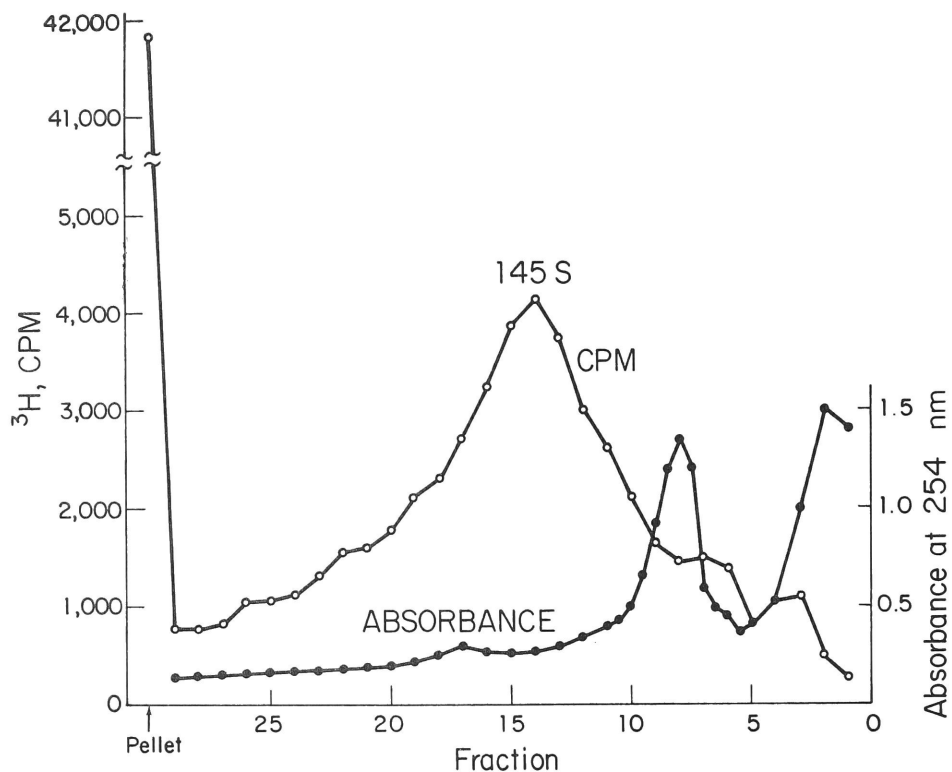


Fig. 33. Distribution of Semliki Forest virus nucleocapsids in a 5-20% (w/w) sucrose gradient in RSB. Centrifugation time: 2 hours. The material centrifuged was an RSB homogenate of infected cells, which had been clarified at low speed. Radioactivity at 145 S (calculated with reference to the single ribosome peak at 75 S) represents viral RNA within nucleocapsids.

TABLE III

Effect of Deoxycholate or EDTA on Distribution in 5-20% Sucrose
Gradient of Structures Containing Labeled Viral RNA

Region of gradient	Approximate S value	³ H in region, percent of total ³ H in RSB gradient		
		RSB	Deoxycholate	EDTA
Top 1/3	0-100 S	13	18	53
Middle 1/3 ^a	100-200 S	32	29	27
Bottom 1/3	200-300 S	11	13	6
Pellet	> 300 S	44	8	21
Total		100	68	107

^a Contains nucleocapsids

The 5-20% sucrose gradient was made up in the same buffer. The results are also shown in Table III. The pellet from the EDTA gradient contains only one-half as many counts as are present in the pellet from the RSB gradient, and there is an increased amount of label in the top one-third of the EDTA gradient. The number of counts in the middle one-third of the gradient, where the 145 S peak of nucleocapsids should be, is not significantly reduced. However, the 145 S peak can no longer be clearly distinguished from the large amounts of radioactivity near the top of the gradient.

The labeled viral RNA released from rapidly-sedimenting structures by deoxycholate or EDTA treatment most likely arises from RNA-replicating structures and viral polyribosomes, both of which are thought to be bound to membranes in infected cells (Friedman, 1968a; Kaariainen and Gomatos, 1969). Neither treatment increases the yield of nucleocapsids above that found in RSB homogenates of infected cells. Thus the RSB homogenization procedure was adopted.

e. Further purification of nucleocapsids. Purification of the nucleocapsids isolated on the 5-20% sucrose gradient was attempted by resedimentation in a sucrose gradient made with D₂O (heavy water) as a solvent instead of water. D₂O was originally used in an attempt to increase the density of the sucrose solution above the buoyant density of the nucleocapsids, to allow equilibrium banding of the nucleocapsids. However, it became apparent that this was not possible, for the density of the nucleocapsids proved higher than expected. Better separations were achieved by using D₂O, possibly because of the increased viscosity of D₂O-sucrose solutions, and therefore its use was continued.

The pooled fractions from the 145 S region of a 5-20% gradient of an infected, ³H-uridine labeled cell homogenate were layered over a 30-40% sucrose gradient made in RSB and D₂O and centrifuged in the SW-27 rotor at 90,000 x g for 15 hours at 4 C. The gradient was collected and analyzed as above, except that 50% (w/w) potassium tartrate was used to displace the dense sucrose solutions. The results are shown in Fig. 34. The radioactivity is confined to a relatively narrow, monodisperse peak; however, the absorbance profile reveals that a number of unlabeled ultraviolet-absorbing components are present in the same

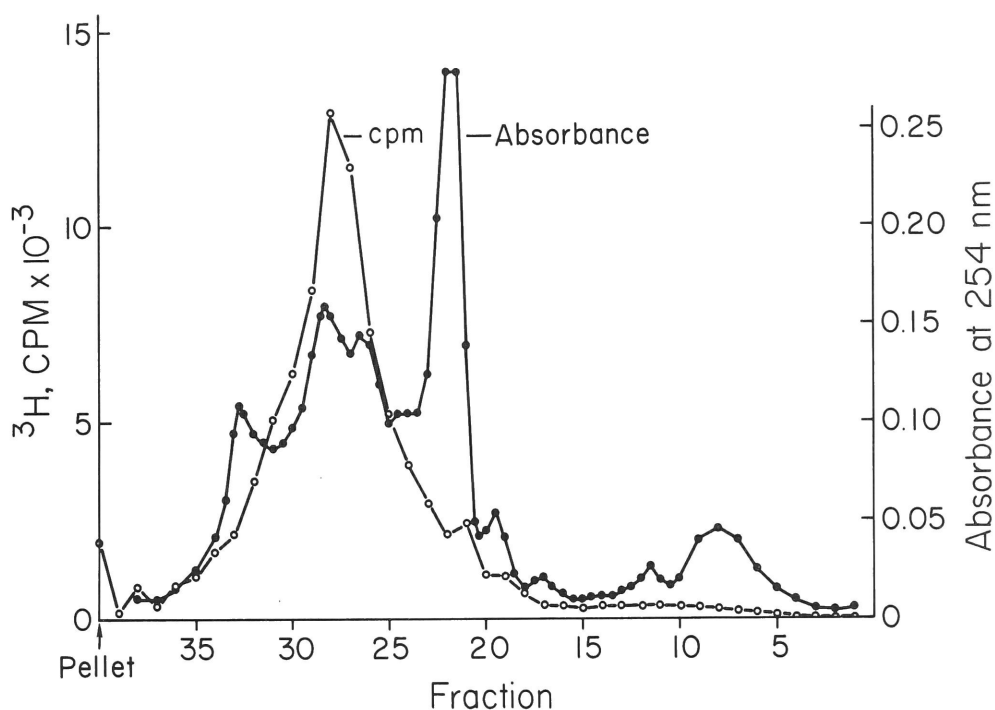


Fig. 34. Distribution of nucleocapsids in a 30-40% sucrose gradient prepared in RSB with D_2O . Centrifugation time: 15 hours. The material centrifuged was from the 145 S region of a 5-20% sucrose gradient similar to that shown in Fig. 33. The peak of radioactivity represents nucleocapsids; the absorbance peaks probably represent the following: at fraction 22, single ribosomes; at fraction 26, ribosome dimers; at fraction 28, nucleocapsids; and at fraction 33, ribosome tetramers.

region as the peak of label. All of these components have high ratios of absorbance at 260 vs 280 nm, indicating that they contain RNA; the positions of the peaks, and the electron microscopic appearance of the material suggested that the four main absorbance peaks in the lower half of the gradient are, respectively, ribosome monomers, ribosome dimers, nucleocapsids (associated with radioactivity) and ribosome tetramers.*

To avoid contamination of nucleocapsids with the various ribosome species, pooled fractions from the 145 S region of the 5-20% sucrose gradient were dialyzed overnight vs. PN buffer (0.01 M sodium phosphate, pH 7.3, 0.1 M NaCl), which lacks Mg^{++} ions, and fractionated again. Sedimentation of the dialyzed material through a 30-40% sucrose gradient prepared in PN with D_2O (Fig. 35) revealed that all ribosome species are dissociated into 60 S and 40 S subunits (absorbance profile). The bulk of the radioactivity in nucleocapsids is associated with a single ultra-violet-absorbing peak which sediments more rapidly than the ribosomal subunits.

A third sedimentation in sucrose was done to concentrate the purified nucleocapsids and to remove traces of contaminating ribosomal material. The peak fractions of nucleocapsids from the 30-40% gradients were pooled, diluted 1:2 in PN buffer, and layered over a 40-45% sucrose gradient in PN made in D_2O . After centrifugation for 12 hours at 90,000 x g, all the labeled nucleocapsids are found in a monodisperse peak which also contains most of the ultraviolet-absorbing material (Fig. 36). A small amount of absorbing material sediments more slowly. The material from the main peak was dialyzed extensively against PN buffer to remove sucrose, and concentrated by dusting the filled dialysis bag with dry Sephadex G-200 and allowing the Sephadex to absorb water. The material in the dialysis bag served as the purified nucleocapsid preparation.

f. Summary of purification procedure. Cells are harvested by scraping 12 hours after infection, pelleted, and vigorously Dounce homogenized in 2 ml cold RSB per 3×10^8 cells. Nuclei and debris are pelleted at

* Ribosome tetramers arranged in parallel sheets (see Byers, 1967) were seen in thin section of infected cells late in the growth cycle of Semliki Forest virus: see part II of this thesis and Grimley et al. (1968).

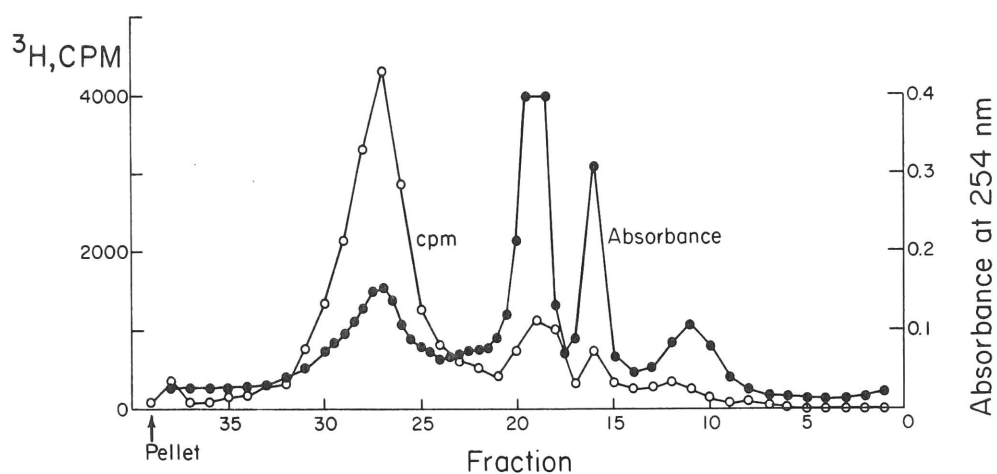


Fig. 35. Distribution of nucleocapsids in a 30-40% sucrose gradient prepared in PN buffer with D_2O . Centrifugation time: 15 hours. The material centrifuged was from the 145 S region of a 5-20% sucrose gradient similar to that shown in Fig. 33. The peak of radioactivity represents nucleocapsids; the absorbance peaks represent the following: at fraction 16, 40 S ribosomal subunits; at fraction 19, 60 S ribosomal subunits; at fraction 27, nucleocapsids.

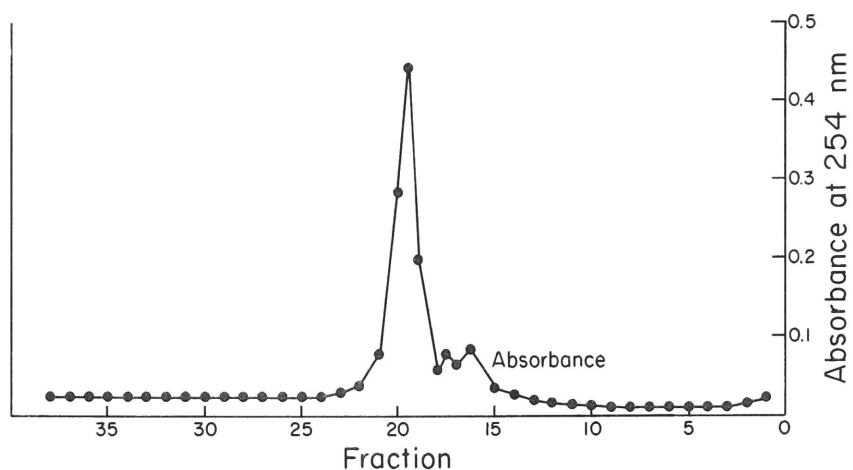


Fig. 36. Distribution of nucleocapsids in a 40-45% sucrose gradient prepared in PN buffer with D_2O . Centrifugation time: 12 hours. The material centrifuged was the nucleocapsid peak from a 30-40% sucrose gradient similar to that shown in Fig. 35. The major peak of absorbing material represents purified nucleocapsids.

1000 x g for 15 min, and the supernatant is layered over a 28 ml 5-20% (w/w) sucrose gradient prepared in RSB. The gradient is centrifuged in the SW 25.1 rotor at 55,000 x g for 2 hr at 4 C, and 1 ml fractions are collected by displacement. The fractions containing the nucleocapsids (usually fractions 11 to 20) are pooled and dialyzed overnight vs 2 changes of 2 liters each of PN buffer. The dialyzed material (12 ml) is layered over a 26 ml 30-40% sucrose gradient prepared in PN with D₂O. The gradient is centrifuged in the SW 27 rotor at 90,000 x g for 15 hr at 4 C, and 1 ml fractions are collected. The fractions containing the nucleocapsids are pooled, diluted 1:2 with PN buffer, and layered over a 26 ml 40-45% sucrose gradient made in PN and D₂O. This is centrifuged in the SW 27 rotor at 90,000 x g for 12 hr at 4 C. One ml fractions are collected; the fractions containing the nucleocapsid peak are pooled, and dialyzed vs 3 changes of 2 liters each of PN buffer for a total of 36 hr. Dry Sephadex G-200 is then dusted over the bag and allowed to absorb water over a period of 1-2 hours, in order to concentrate the nucleocapsids.

The yield of purified nucleocapsids from 10^9 infected cells (100 100 mm Petri dishes) is approximately 150 µg. Approximately 1/3 of the ³H-uridine radioactivity present in the 145 S peak from the first sucrose gradient is recovered in the purified preparation. Thus, on the order of 450 µg of nucleocapsids are originally present in 10^9 cells, or approximately 18,000 nucleocapsids per cell (using an estimate of 15×10^6 daltons for the particle weight per nucleocapsid: see Appendix). In terms of protein, the nucleocapsids are purified roughly 125-fold from the original clarified cell homogenate.

2. Purity of Nucleocapsid Preparations

To determine the purity of nucleocapsids prepared in the manner described above, homogenates of uninfected chick embryo cells labeled with either ³H-leucine or ³H-uridine were mixed with homogenates or unlabeled, infected cells. Nucleocapsids were purified from the mixture, and the specific activities of the original homogenates and the purified nucleocapsid preparations were determined (Table IV). Since the nucleocapsids were made in the absence of label, the ratio of the specific

TABLE IV

Homogenates of uninfected cells, grown in the presence of either ^3H -uridine or ^3H -leucine, were mixed with homogenates of infected, unlabeled cells, and nucleocapsids were purified by successive sedimentation in 2 or 3 sucrose gradients, as described in the text. Specific activities of the purified nucleocapsid preparations and the original homogenates were determined, and their ratio calculated, to estimate percent contamination.

-
- ^a Nucleocapsids were treated with Triton X-100, as follows:
 a portion of a dialyzed pool of nucleocapsids partially purified in a 5-20% sucrose gradient was treated with 0.5% Triton X-100 (Ruger Chemical Co., Irvington-on-Hudson, N.Y.) at 0°C, immediately layered over a 35-38% sucrose gradient, and centrifuged for 15 hours. Another portion of the pool was not treated with the detergent.
- ^b Nucleocapsids were treated with DNase, as follows: 2 mM MnCl_2 and 10 $\mu\text{g/ml}$ DNase (electrophoretically pure, Worthington) were added to a dialyzed pool of nucleocapsids partially purified in a 5-20% sucrose gradient. The mixture, in which a precipitate formed, was held at room temperature for 15 minutes. Then 2.5 mM EDTA was added, at which time the solution cleared. The solution was layered over a 30-40% sucrose gradient and centrifuged for 18 hours.

TABLE IV
Purity of Nucleocapsid Preparations

Labeled macro- molecule from uninfected cells	Experi- ment	Number of successive sucrose gradients in purification procedure	Specific activity, cpm/ μ g RNA or protein		
			Cell homogenate (labeled uninfected plus unlabeled infected)	Purified Nucleo- capsid	Percent contami- nation
RNA	A	2	5500	660	12%
	B	2	6100	350	6%
	C	3	6700	360	5%
Protein	B	2	930	270	29%
	C	3	615	180	29%
	D	3	800	218	27%
	E	2	500	128	25%
	E ^a	2	500	143	29%
	F ^b	2	470	98	21%

a, b See legend.

activity of the purified nucleocapsids to that of the original mixture should correspond to the percent contamination of the nucleocapsid preparation with normal cellular protein or RNA.

Table IV shows that only 5% of the RNA in purified nucleocapsid preparations is of cellular origin after purification in 3 successive sucrose gradients. However, approximately 25-30% of the protein in purified nucleocapsid preparations is cellular in origin; this protein was not removed by treatment of partially purified nucleocapsid either with Triton x-100, a nonionic detergent which should disrupt lipoproteins, or with deoxyribonuclease, which should destroy DNA-protein complexes. This contaminating protein was clearly associated with the peak of purified nucleocapsids in the third (40-45%) sucrose gradient, as shown in Fig. 37. Most of the labeled contaminating protein also remained associated with purified nucleocapsids banded isopycnically in a potassium tartrate density gradient. These results indicate that the contaminating protein is bound to the nucleocapsids during the isolation and purification procedure. The size distribution of this protein is analyzed in section 9 below.

3. Morphology of Purified Nucleocapsids

Unfixed nucleocapsids negatively stained with phosphotungstate appear poorly contrasted and of irregular outline in the electron microscope. After fixation in 4% formaldehyde for 1 hour at 0 C, a more regular profile is seen (Figs. 38, 39). Little extraneous material is present in purified preparations, indicating that the impurity which constitutes 25% of the protein is probably not particulate. Nucleocapsids are roughly circular in profile and approximately 400 A in diameter. They exhibit a mottled surface morphology indicative of a subunit structure, but no symmetry can be clearly discerned. In some preparations (Figs. 38, 39) a majority of the nucleocapsids are penetrated by the phosphotungstate, giving them the appearance of a hollow shell. However, in other preparations few nucleocapsids are penetrated (Fig. 40). The reason for this variability is at present unknown.

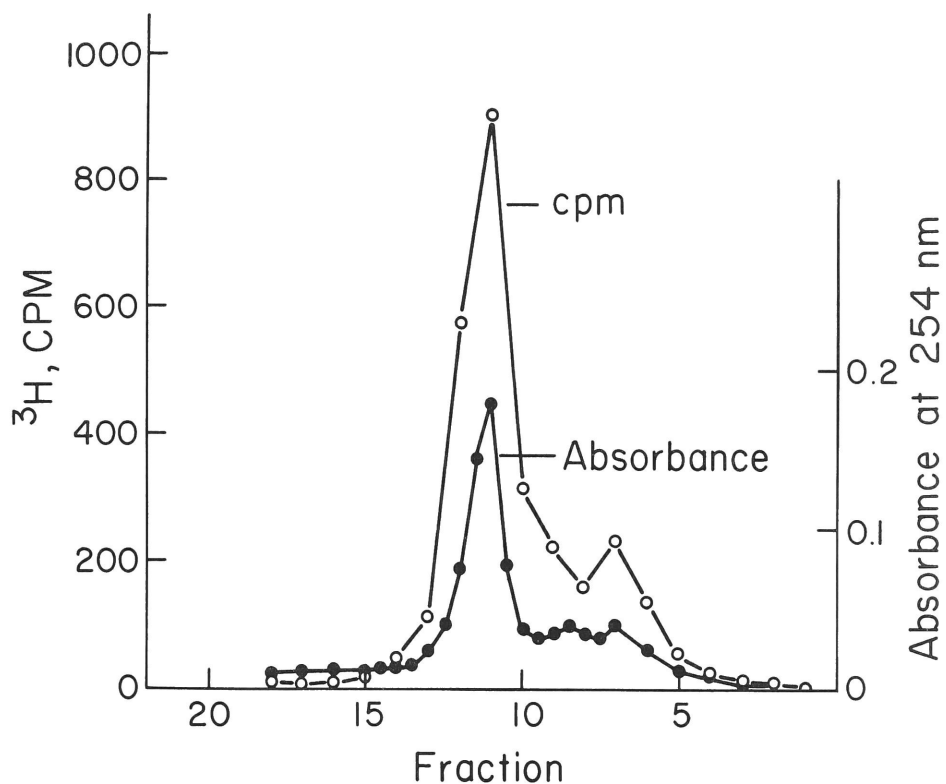


Fig. 37. Cosedimentation of contaminating cellular protein and purified nucleocapsids in a 40-45% sucrose gradient similar to that shown in Fig. 36. Cells labeled for 2 days with ^3H -leucine were grown in unlabeled medium for 12 hours and then infected. Nucleocapsids were harvested and purified by sedimentation in 2 successive sucrose gradients. The purified nucleocapsids were centrifuged into a 40-45% sucrose gradient and the first 18 fractions of the gradient were analyzed for absorbance and radioactivity.

Fig. 38. Fixed, negatively-stained Semliki Forest virus nucleocapsids purified as described in the text. Little extraneous material is present. The particles are of uniform size, with a diameter of approximately 400 A. Most nucleocapsids are penetrated by the phosphotungstate. Magnification: x 148,000.

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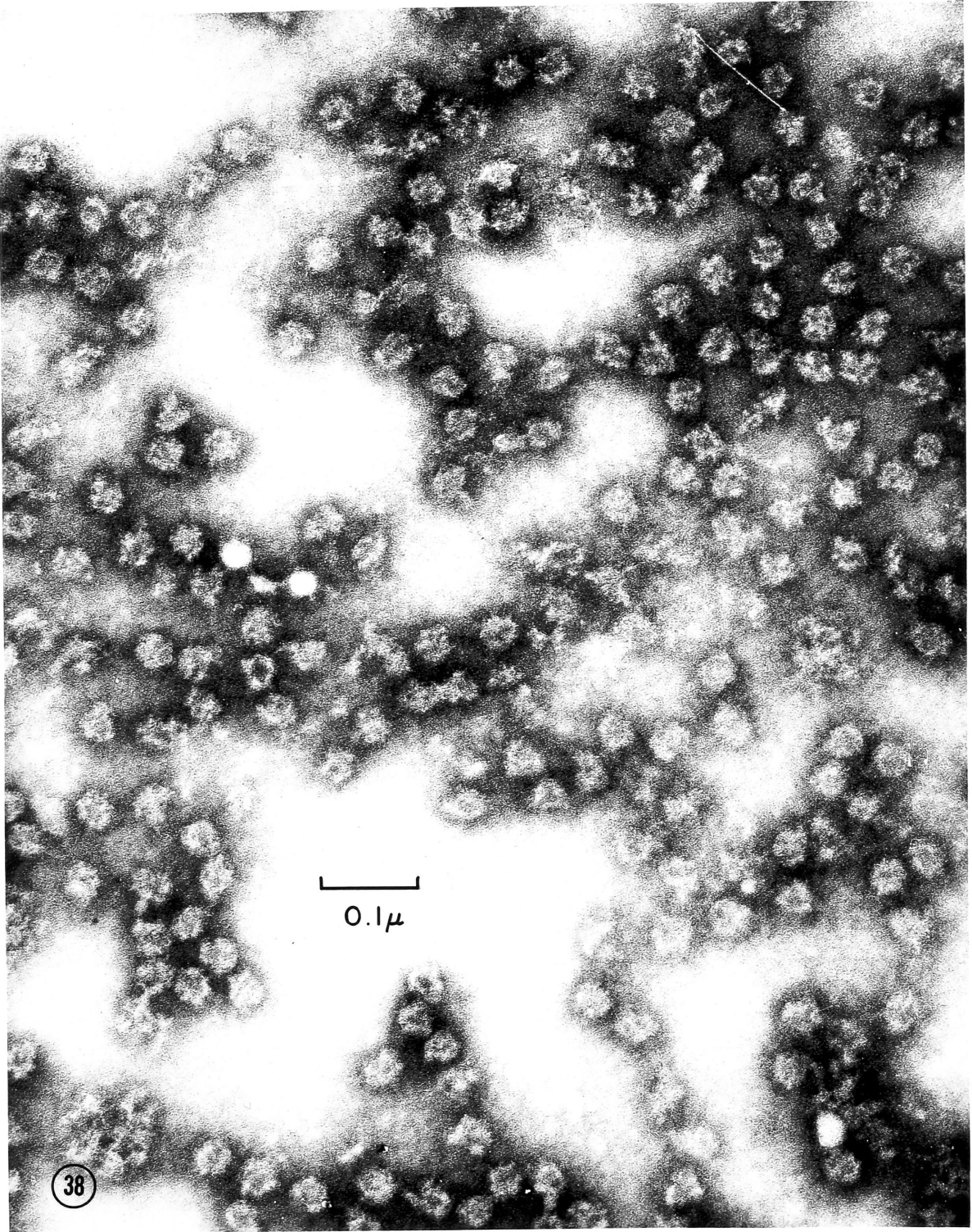


Fig. 39. Fixed, negatively-stained purified nucleocapsids. Photographic enlargement of a view similar to that shown in Fig. 38. The distribution of stain-excluding areas is suggestive of a subunit pattern at the surface of the particles. Magnification: x 260,000.

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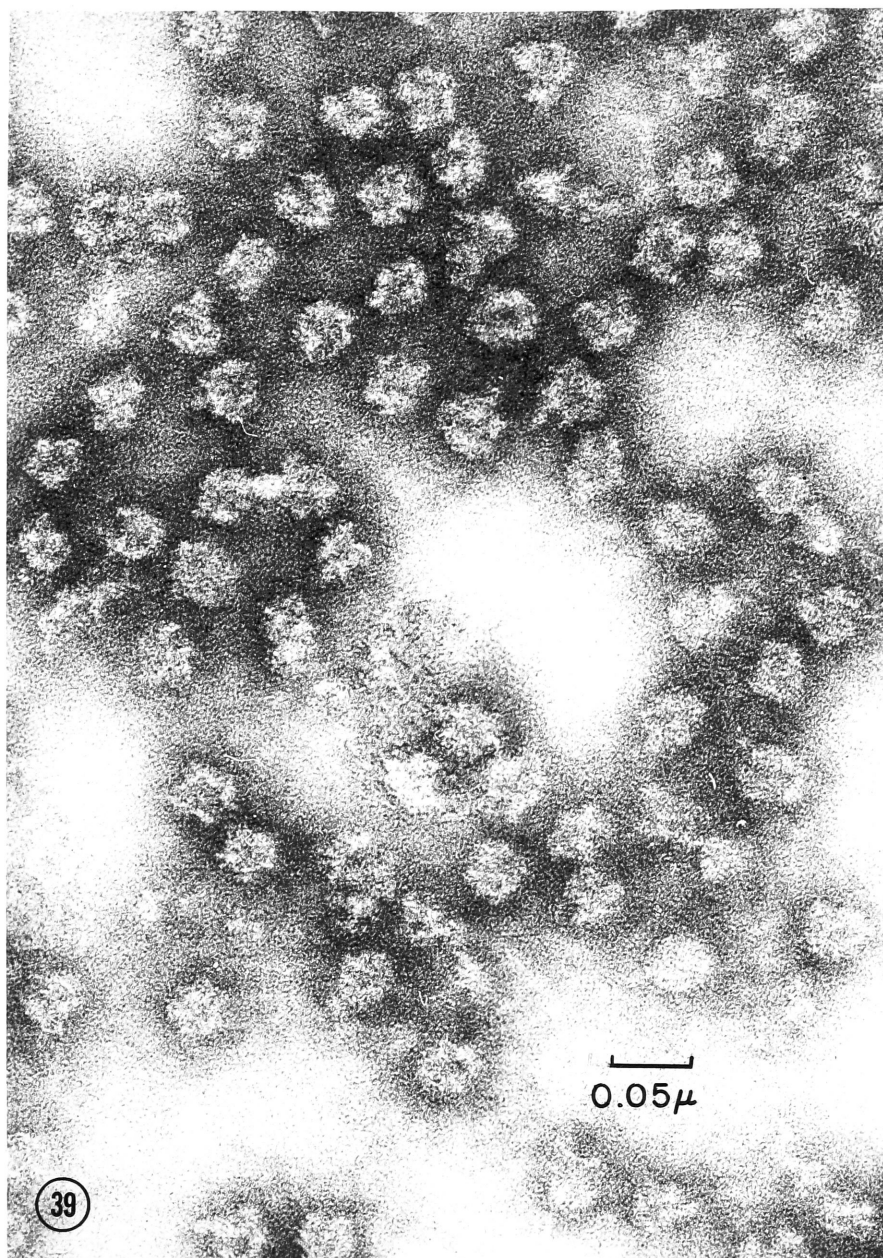
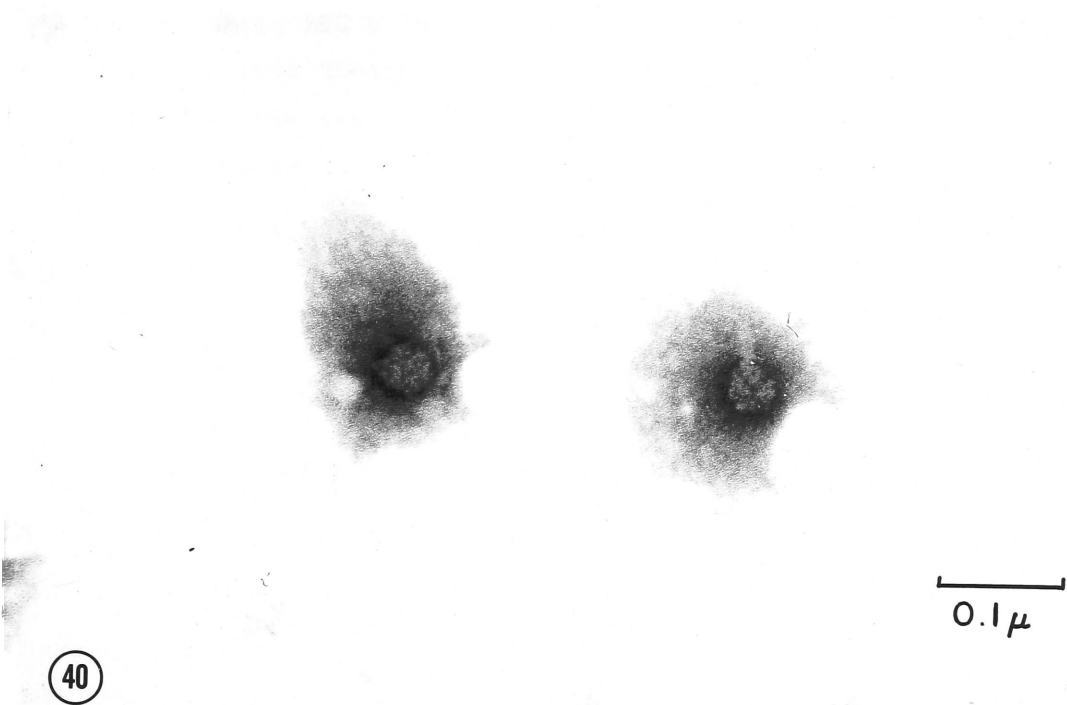


Fig. 40. Purified nucleocapsids which are not penetrated by
the phosphotungstate. Magnification: x 160,000.

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In thin sections of infected cells, virus nucleoids, which probably correspond to the isolated nucleocapsids, appear as densely-staining, spherical shells 280 A in diameter, with a less dense interior (see Fig. 9, Part I of this thesis). An identical structure can be seen in virus particles in thin sections (Figs. 5 and 6, Part I of this thesis; also, Simpson and Hauser, 1968b).

Thus isolated nucleocapsids and nucleocapsids in thin sections of infected cells or virus particles all have the same basic morphology, but negatively-stained nucleocapsids have a somewhat larger diameter than those in thin sections. The reason for this discrepancy in size is unknown, but it may reflect a swelling of the nucleocapsids in the buffers used during the isolation and purification procedure.

4. Size of RNA in Nucleocapsids

Nucleocapsids labeled with ^3H -uridine were partially purified by sedimentation through a single 5-20% sucrose gradient (cf. Fig. 33). The RNA was extracted with 1% SDS and analyzed on a sucrose gradient (Fig. 41). Most of the RNA sediments as a single peak at 45 S; however, there is a variable amount of material sedimenting from 10 to 40 S. Under the same conditions, RNA extracted from purified virus also has a sedimentation coefficient of 45 S (Fig. 42). In nucleocapsids which have been more extensively purified, the amount of slower sedimenting RNA is increased in relation to 45 S RNA, indicating that there is some breakdown of the RNA within intact nucleocapsids. However, at no time is there any distinct peak other than that at 45 S; the fragmentation of the RNA appears to be nonspecific. The similarity of the RNA in the 145 S structures to that in virus particles strongly supports the idea that these structures are nucleocapsids, which are incorporated into complete virus particles by the acquisition of an envelope, as shown in Part A of this thesis.

Friedman and Berezsky (1967) also found 45 S RNA in partially purified nucleocapsids of Semliki Forest virus. However, Sreevalsan and Allen (1968) reported that in addition to the viral (40 S) RNA, some 26 S RNA was present in nucleocapsids of western equine encephalitis virus obtained by centrifugation of infected cell extracts on sucrose

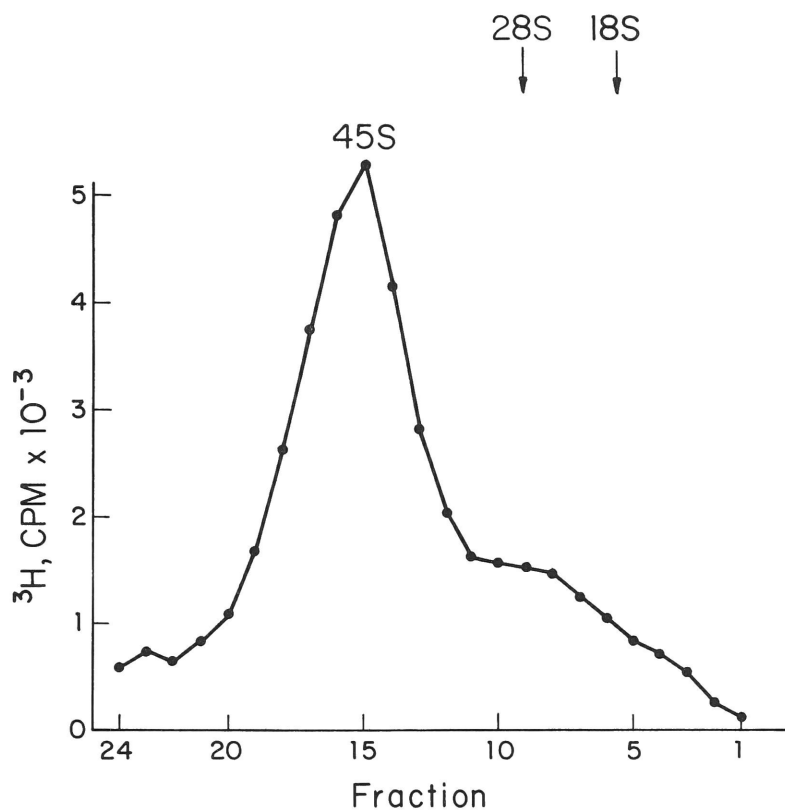


Fig. 41. Sedimentation analysis of viral RNA extracted from nucleocapsids partially purified in a single 5-20% sucrose gradient. The S value of viral RNA was determined from the positions of marker chick embryo ribosomal RNA's.

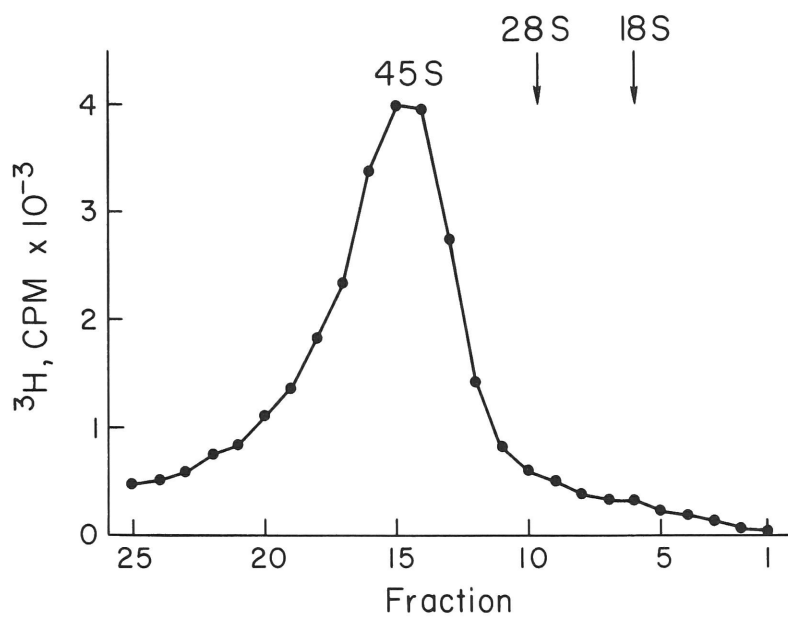


Fig. 42. Sedimentation analysis of the RNA extracted from Semliki Forest virus purified as described in Materials and Methods.

gradients. It is possible that the 26 S RNA was derived from a 65 S structure present in sucrose gradients of infected cell extracts. The 65 S structure, whose exact nature is not known, contains 26 S RNA.

5. Ribonuclease Sensitivity of RNA Within Nucleocapsids

The RNA within Semliki Forest virus nucleocapsids can be digested to acid-soluble fragments by relatively mild digestion with pancreatic ribonuclease. A cytoplasmic extract of infected, Actinomycin D-treated cells labeled from 1 to 12 hours after infection with ^3H -uridine was divided into two halves. One half was incubated with 1 $\mu\text{g}/\text{ml}$ ribonuclease for 10 minutes at 37 C, and the other half was incubated under the same conditions without enzyme. Each extract was layered over a 5-20% sucrose gradient and centrifuged for 2 hours at 55,000 x g, and the acid-precipitable radioactivity in each fraction was determined (Fig. 43). Under these conditions approximately 50% of the ^3H -labeled RNA counts present in the 145 S peak in the untreated extract are lost in the ribonuclease-treated sample. Thus some of the RNA within the nucleocapsids has been digested by ribonuclease treatment.

The peak of label which remains after ribonuclease treatment has the same sedimentation rate as that in the untreated sample. This raised the possibility that only a fraction of the nucleocapsids, as isolated, is sensitive to ribonuclease and the remainder is resistant. To test this possibility, a preparation of ^3H -uridine-labeled nucleocapsids, partially purified by sedimentation into a 5-20% sucrose gradient, was incubated with 1 $\mu\text{g}/\text{ml}$ ribonuclease at 37 C, and the acid-precipitable label remaining after varying times was measured. From the results, shown in Fig. 44, it is clear that on prolonged incubation virtually all of the RNA contained within nucleocapsids is rendered acid-soluble by ribonuclease digestion. Thus there is not a population of nucleocapsids which is resistant to ribonuclease treatment. It should be noted that in this experiment, in which partially purified nucleocapsids were treated with ribonuclease, 94% of the RNA was digested to acid-soluble fragments after 10 minutes at 37 C. In the experiment in which ribonuclease digestion was carried out on the crude cytoplasmic extract (Fig. 43) only 50% of the RNA in nucleocapsids

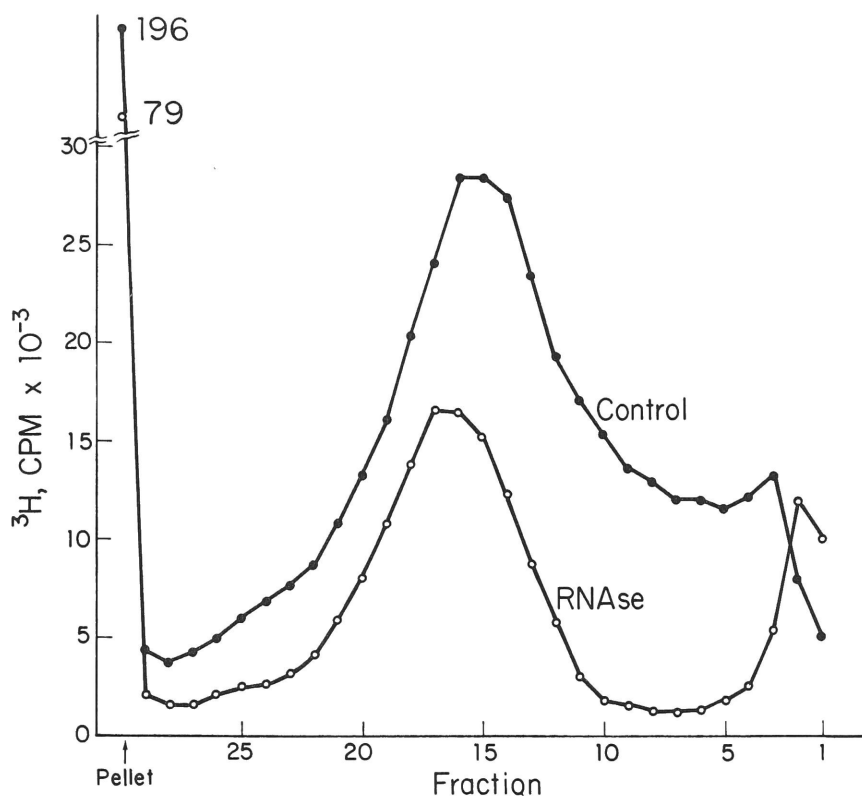


Fig. 43. Sensitivity of the RNA within nucleocapsids to digestion by ribonuclease. Filled circles: acid-precipitable radioactivity in structures containing viral RNA from untreated infected cell homogenate. Open circles: radioactivity from homogenate incubated 10 minutes at 37 C with 1 μ g/ml pancreatic ribonuclease. Homogenates were centrifuged in identical 5-20% sucrose gradients for 2 hours.

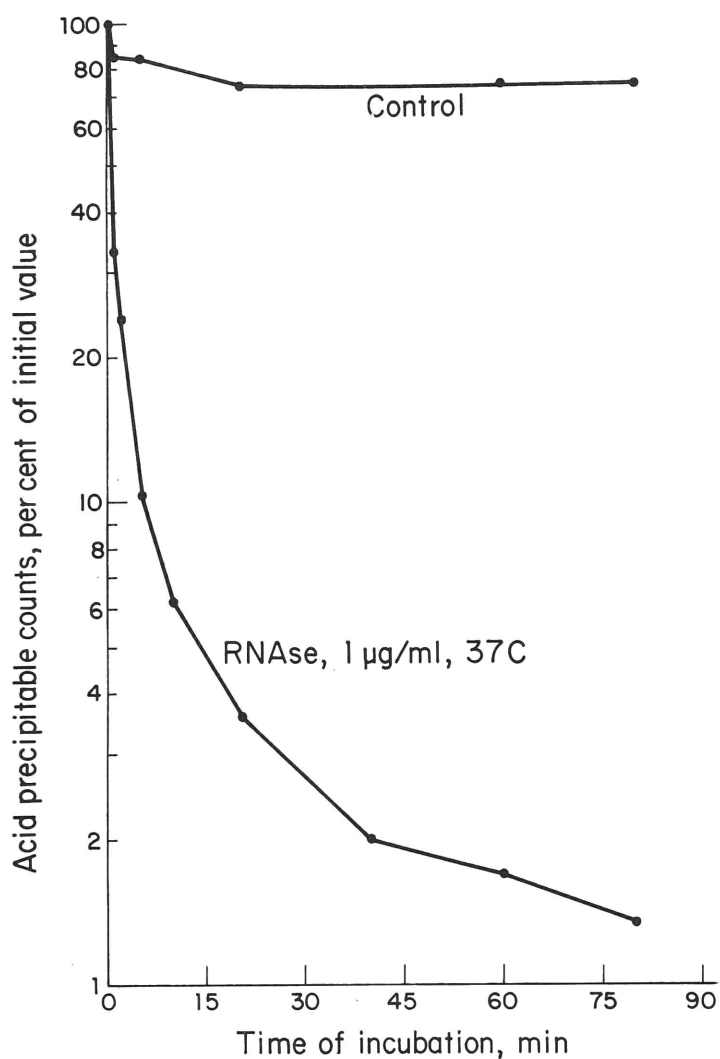


Fig. 44. Digestion of the RNA within nucleocapsids by ribonuclease. Partially purified nucleocapsids were used. Acid-precipitable radioactivity in RNA was determined at various times by adding 250 µg of bovine serum albumin and 1.5 ml ice cold 10% trichloroacetic acid to one-tenth ml aliquots of nucleocapsids. The samples were stored at 0 C, the precipitate collected on Whatman GF/C filters, dried in an oven and counted in toluene-Liquifluor scintillation fluid.

was digested. This may have been due to the presence of large amounts of cellular RNA in the crude extract.

The accessibility of the RNA within nucleocapsids to ribonuclease may explain the appearance of some slower-sedimenting RNA in more highly purified nucleocapsids (see section 4). The purification procedure, although carried out at 4 C, requires several days, and any nucleases which might contaminate the nucleocapsid preparations would have the opportunity to break down some of the 45 S RNA into smaller fragments.

The RNA within nucleocapsids of two other group A arboviruses, western equine encephalitis virus (Sreevalsan and Allen, 1968) and Sindbis virus (Strauss et al., 1968), has also been reported to be accessible to digestion by ribonuclease, but Friedman and Berezsky (1967) reported that Semliki Forest virus nucleocapsids are not ribonuclease-sensitive. The latter authors carried out an experiment nearly identical to that shown in Fig. 43, in which a ribonuclease-treated infected cell homogenate was analyzed on a sucrose gradient. They found a peak of ^3H -labeled material at 145 S in such a gradient, and concluded that the 145 S material was not sensitive to the enzyme. However, they did not report the results of controls in which the homogenate was not incubated with ribonuclease. Therefore it is not known how much label was in the 145 S peak before ribonuclease treatment. It is probable that ribonuclease digested some of the RNA within nucleocapsids in their experiment as it clearly did in the experiment shown in Fig. 43.

6. Fate of Ribonuclease-treated Nucleocapsids

When nucleocapsids purified by three successive sucrose gradient sedimentations were incubated with 1 $\mu\text{g}/\text{ml}$ ribonuclease for 10 minutes at 37 C, then fixed in formaldehyde and negatively stained, very few intact nucleocapsids were visible in the electron microscope. Controls incubated under the same conditions without enzyme, then fixed and negatively stained, revealed numerous images of nucleocapsids, as in Fig. 38. Although the technique of negative staining does not lend itself well to quantitation, these results indicate that most of the nucleocapsids disintegrate under the above conditions of ribonuclease digestion.

7. Proteins of Purified Nucleocapsids and Virus

Gel electrophoresis was carried out on proteins solubilized from purified nucleocapsids and purified virus labeled with a mixture of ^3H -amino acids from 4 to 9 hours after infection. Actinomycin D (2 $\mu\text{g}/\text{ml}$) was present from the time of infection until 4 hours to reduce cellular protein synthesis. Under these conditions most of the labeled proteins synthesized are of viral, rather than cellular, origin (Friedman, 1968a; Strauss et al., 1969).

SDS-polyacrylamide gels of proteins from purified nucleocapsids display a major and a minor peak of labeled protein, denoted 1 and 3 in Fig. 45. Protein 1 migrates more rapidly through the gel than protein 3. The ratio of label in peak 3 to that in peak 1 is approximately 0.15:1.

Gels of proteins from purified virus (Fig. 46) display, in addition to peaks 1 and 3, a single major component which migrates at an intermediate rate and is designated protein 2. The ratio of label in peak 2 to that in peak 1 is approximately 4:1. The ratio of label in peak 3 to that in peak 1 is 0.18:1. This value is not significantly different from the same ratio in gels of purified nucleocapsids. This result implies that nucleocapsid proteins are incorporated into virus particles in the same proportion as they are present in the unenveloped nucleocapsid.

Gels run in parallel to those analyzed for radioactivity were stained with Coomassie blue. Trypsin and bovine serum albumin were included in some gels as markers with known molecular weights. The rate of migration of a denatured, reduced polypeptide chain in an SDS-polyacrylamide gel is inversely proportional to the logarithm of its molecular weight (Shapiro et al., 1967). By comparing the mobilities of the three viral proteins in stained gels with those of trypsin and bovine serum albumin, the molecular weights of the viral proteins may be estimated to be: protein 1, 35,000; protein 2, 55,000; protein 3, 70,000. These molecular weight estimates are only approximate, for there is some variability in the mobility of a given protein in different gels even when run at the same time. Additionally, the relation between mobility and molecular weight is only empirically established, and it is possible that certain proteins may not behave as predicted.

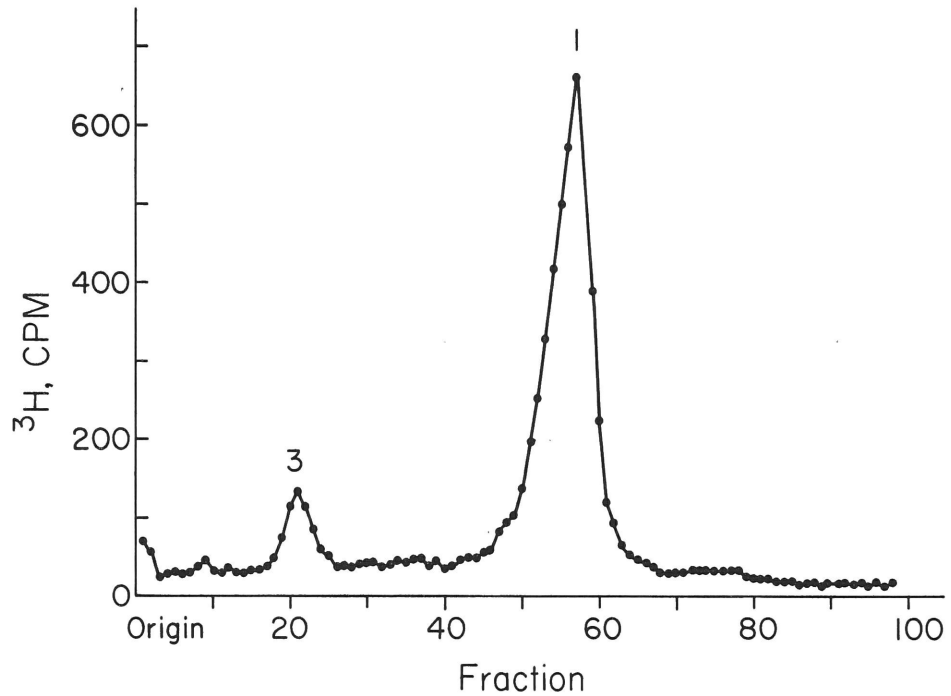


Fig. 45. SDS-polyacrylamide gel electrophoresis of proteins from purified nucleocapsids, labeled from 4 to 9 hours after infection with ^3H -amino acid mixture. 7.5% gels were run at 3 volts/cm for 14 hours. Each point represents the radioactivity in a 1-mm slice of the frozen gel. Peaks 1 and 3 are the major and minor nucleocapsid proteins.

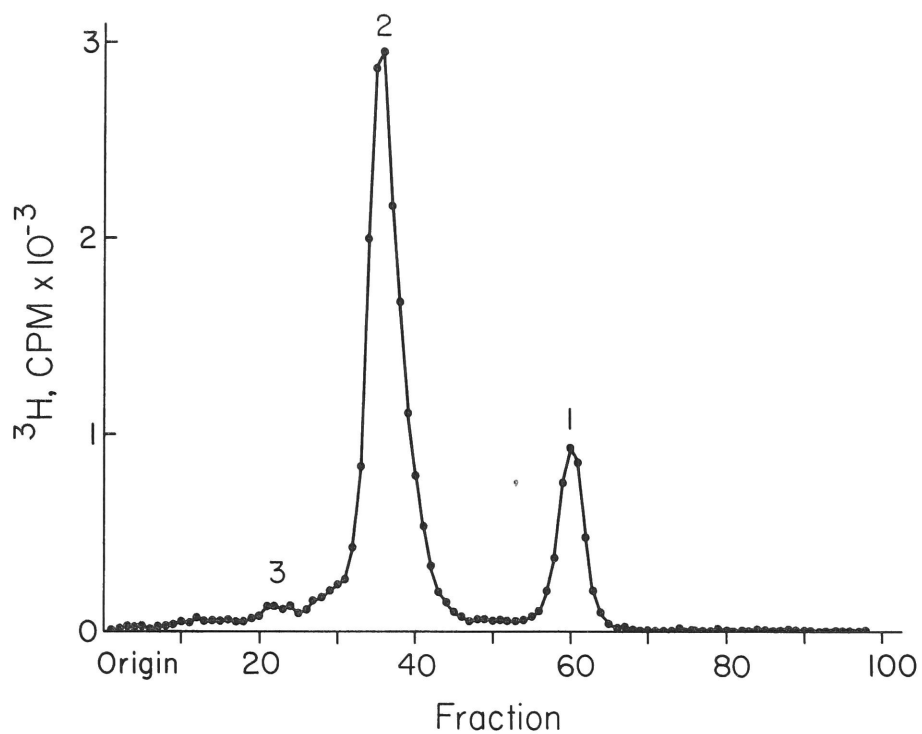


Fig. 46. Polyacrylamide gel electrophoresis of proteins from purified virus, labeled from 4 to 9 hours after infection with ^3H -amino acid mixture. Peaks 1 and 3 are the nucleocapsid proteins: peak 2 is the envelope protein.

It should be noted that the estimated molecular weight of the minor nucleocapsid protein (No. 3) is just twice that of the major nucleocapsid protein (No. 1). It is possible that the material in peak 3 is simply a dimer of the protein present in peak 1. To investigate this possibility, it is planned to determine the relative content of certain amino acids in each of the peaks. If there are significant differences in amino acid composition between the two peaks, their identities as separate proteins will be established.

It is not known, however, whether there may be more than one species of polypeptide chain under any of the three peaks. Since SDS-acrylamide gels separate proteins almost exclusively on the basis of molecular weight, two distinct proteins of the same molecular weight would have nearly the same mobilities and might show up as a single peak. Other methods, such as end-group or immunological analysis, are necessary to determine conclusively how many different proteins are present.

From the molecular weights and relative proportions of the three virus proteins, and an estimate of the total mass of protein per virus particle, it can be calculated (see Appendix) that there are approximately 280 molecules of protein 1 (the major nucleocapsid protein), 710 of protein 2 (the envelope protein), and 20 of protein 3 (the minor nucleocapsid protein) per virus particle. The molecular weight of the nucleocapsid can be estimated to be 16 million daltons. As described in the Appendix, the accuracy of these values is dependent on the value of the molecular weight of the viral RNA, which is not known with certainty.

Friedman (1968a) also reported that Semliki Forest virus nucleocapsids contain two proteins, and virus particles contain three proteins. The relative amounts of each protein were not reported, for the bands of radioactive protein in SDS-polyacrylamide gel electropherograms were detected by autoradiography, which is not a quantitative technique. Hay et al. (1968) found only two radioactive bands in gels of purified Semliki Forest virus labeled with various amino acids. One of these, which corresponds to protein 2, was also found in a hemagglutinating

particle which has a lower density than whole virus and probably consists of empty viral envelopes. In stained gels of purified virus proteins, however, a third band, which migrated slightly slower than the envelope protein, was discerned. It seems likely that this is protein 3, which was missed on the radioactivity profiles because it is present in very small proportion to protein 2, and may simply show up as a shoulder on peak 2 as is apparent in their Figs. 3 and 5 (Hay et al., 1968).

Sindbis virus, which is closely related to Semliki Forest virus, has been reported to contain only two major proteins by gel electrophoresis (Strauss et al., 1968). In this case, nucleocapsids derived from virus particles by detergent treatment were shown to contain only one protein. This apparent chemical difference between the nucleocapsids of Semliki Forest virus and Sindbis virus is paralleled by a morphological difference in the nucleocapsids in thin sections of virus particles (Simpson and Hauser, 1968b). However, it is not known whether these two features are related.

8. Electrophoretic Analysis of the Proteins Which Contaminate Purified Nucleocapsids

Once the electrophoretic pattern of viral proteins in purified nucleocapsids had been determined on polyacrylamide gels, it was of interest to analyze the cellular protein which contaminates the nucleocapsid preparations to the extent of 25% of the total protein. Cell monolayers were grown for 2 days in the presence of 2 μ C/ml 3 H-leucine, in reinforced Eagle's medium containing one-fourth the normal leucine concentration and 2% calf serum. The medium was removed, unlabeled lactalbumin medium added to the cells, and incubation was continued for 12 hours. Cells were then washed and infected as usual, and the infected cells were incubated with unlabeled reinforced Eagle's medium with normal leucine concentration. Most of the 3 H-leucine should have been utilized or washed out during the 12-hour period prior to infection and whatever radioactive leucine was made available to the infected cells by turnover of labeled cellular proteins should have been diluted out by the relatively high leucine concentration in the reinforced Eagle's medium.

At nine hours after infection, cells were harvested and nucleocapsids were purified as described above. Electrophoresis was carried out on the solubilized proteins, and the radioactivity profile from such a gel is shown in Fig. 47. The irregularity of the pattern is probably due to the very low number of counts per slice, leading to a large counting error. There are no clearly distinguishable major peaks of labeled cellular protein in the purified nucleocapsid preparation. Most of the counts are located towards the origin. This would be expected for a random mixture of proteins with a wide range of molecular weights. It thus appears that the cellular proteins which are found in purified nucleocapsid preparations are of many different molecular weights, and do not represent one or a few species.

9. Ultraviolet Absorption Spectrum of Purified Nucleocapsids

In Fig. 48 is shown the absorption spectrum from 225 to 290 nm of purified nucleocapsids in PN buffer. The profile is typical for a ribonucleoprotein, with a minimum at 240 nm and a maximum at 258-260 nm. The ratio of absorbance at 260 nm to that at 280 nm ranges from 1.75 to 1.80 in various preparations. This ratio, which is an approximate measure of the RNA content of ribonucleoproteins, has a value of 1.65 to 1.70 for most enteroviruses, including the well-characterized poliovirus (Schaffer and Schwerdt, 1965). Enteroviruses contain from 20 to 30% RNA (Schaffer and Schwerdt, 1965); thus Semliki Forest virus nucleocapsids, with a higher 260/280 ratio, may have an RNA content substantially greater than 30% of their total mass.

10. Buoyant Density of Nucleocapsids in Potassium Tartrate and Cesium Chloride Gradients

Nucleocapsids form a narrow band at a density of 1.34 gm/cc if centrifuged into a preformed gradient of potassium tartrate. When the nucleocapsids are labeled with ^3H -uridine, all the label is associated with this band (Fig. 49). However, RNA extracted from tartrate-banded nucleocapsids after dialysis is found to be fragmented into predominantly 5 S to 30 S acid-insoluble fragments (Fig. 50). This breakdown cannot be inhibited by autoclaving the potassium tartrate solutions for 40 minutes at 121 C prior to preparing the gradients. The reason

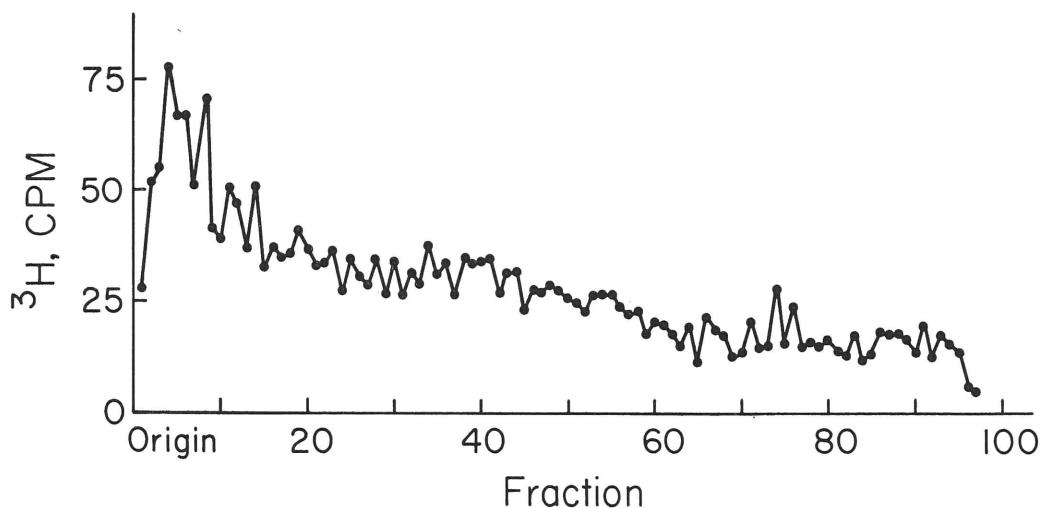


Fig. 47. SDS-polyacrylamide gel electrophoresis of labeled cellular proteins which contaminate purified nucleocapsids. Cells were grown for 2 days in the presence of ^3H -leucine, then for 12 hours in unlabeled medium. Cells were infected, incubated for 9 hours with unlabeled medium, and harvested. Nucleocapsids were purified from the cell homogenate. The labeled cellular proteins in the purified nucleocapsid preparations were denatured and analyzed on an SDS-polyacrylamide gel.

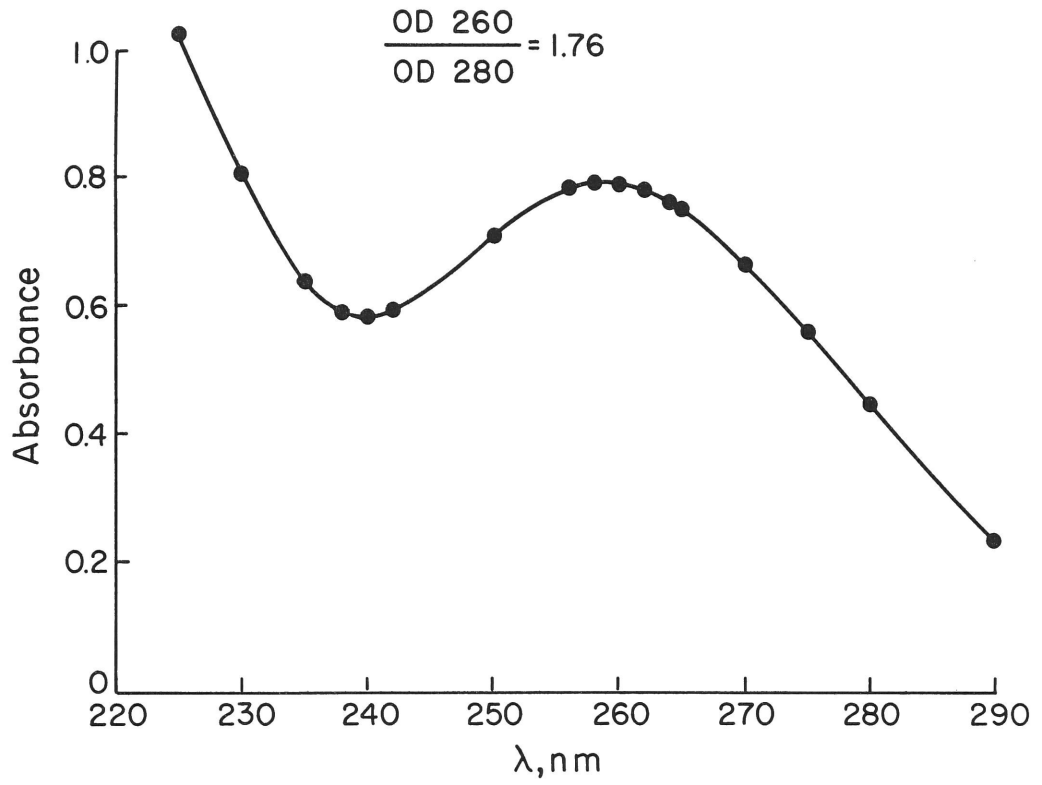


Fig. 48. Ultraviolet absorption spectrum of purified nucleocapsids in PN buffer, determined in a Zeiss PMQ II Spectrophotometer.

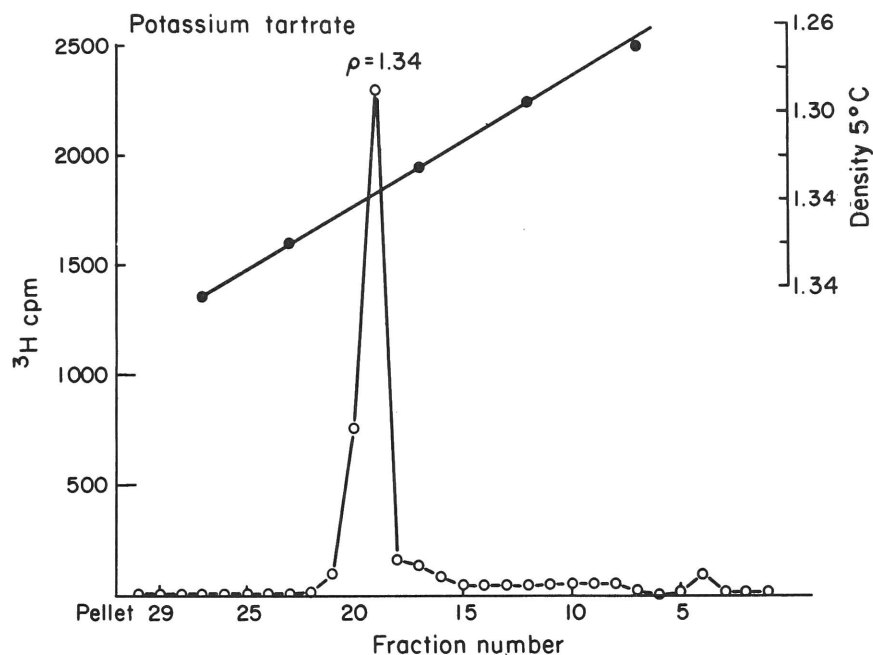


Fig. 49. Buoyant density of nucleocapsids in potassium tartrate density gradient. One ml of ^3H -uridine labeled nucleocapsids, purified by 2 sedimentations in sucrose gradients, was layered over a 29 ml 30-50% (w/w) potassium tartrate gradient in PN buffer and centrifuged in the SW-25 rotor at 55,000 \times g for 15 hours at 5 C. Aliquots of 1 ml fractions were assayed for acid-precipitable radioactivity (open circles). Measurements of the refractive index of selected fractions were made in a Bausch and Lomb Abbe refractometer, and the density of the solutions at 5 C (filled circles) was calculated from an empirically determined relation between refractive index and density.

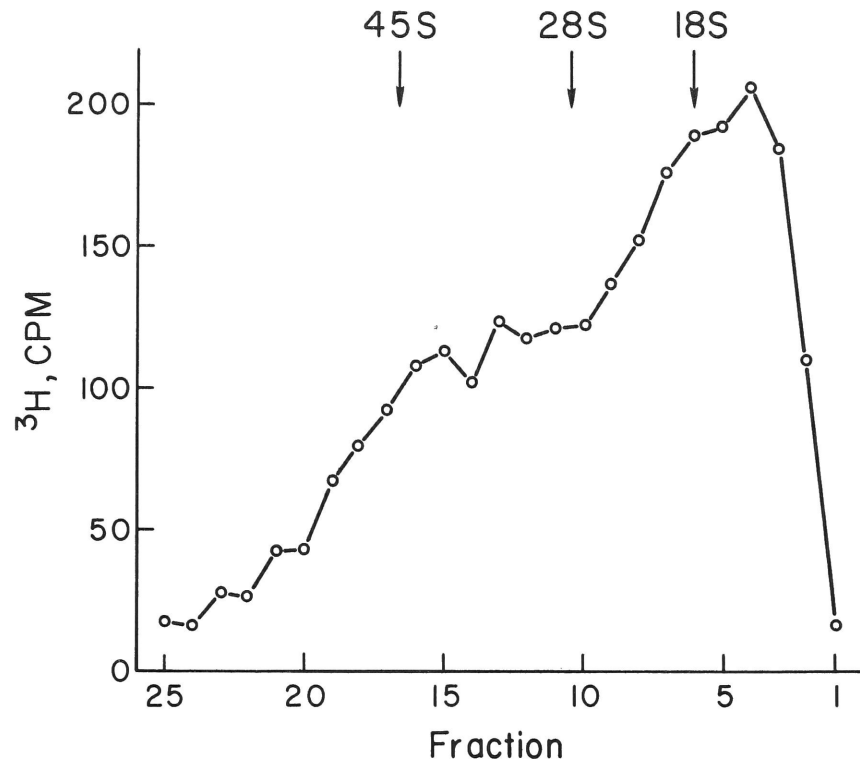


Fig. 50. Sedimentation analysis of labeled RNA extracted from nucleocapsids banded in a potassium tartrate gradient. Nucleocapsids were dialyzed in RSB buffer to remove the potassium tartrate before RNA was extracted.

for the fragmentation of the RNA is not known; however, it precludes the use of tartrate gradients in the purification of nucleocapsids with intact RNA.

When centrifuged in cesium chloride gradients up to a maximum density of 1.55 gm/cc, unfixed nucleocapsids form no visible bands, and essentially all of the radioactivity in the RNA of ^3H -uridine-labeled nucleocapsids is found in the pellet. However, after fixation with formaldehyde the nucleocapsids form a sharp band at a density of 1.47 gm/cc (Fig. 51). All of the labeled RNA is found in this band. Thus unfixed nucleocapsids appear to be disrupted in high concentrations of cesium chloride: the RNA is released and sediments to the bottom of the gradient. Fixation in formaldehyde stabilizes the nucleocapsid structure in cesium chloride gradients, allowing its buoyant density to be determined. This behavior is similar to that of RNA-containing plant viruses such as alfalfa mosaic virus (Hull et al., 1969).

The buoyant densities of Semliki Forest virus nucleocapsids in potassium tartrate ($\rho = 1.34$ gm/cc) and cesium chloride ($\rho = 1.47$ gm/cc) are significantly higher than the buoyant densities of typical enteroviruses, which are as follows: approximately 1.25 gm/cc in potassium tartrate (poliovirus, unpublished observation) and from 1.33 to 1.38 gm/cc in cesium chloride (Schaffer and Schwerdt, 1965). As also suggested by the high ratio of absorbance at 260 nm vs. 280 nm (section 9), the high density of Semliki Forest virus nucleocapsids may reflect a higher RNA content than that of the enteroviruses.

D. Discussion

1. Purification Method

The method developed for the purification of Semliki Forest virus nucleocapsids from infected cell homogenates results in a preparation which is approximately 75% pure in terms of protein, and 95% pure in terms of RNA. This method utilizes no enzymes or detergents, which might alter the nucleocapsids from their native state. The basic attack of the method is to dissociate ribosome species which contaminate nucleocapsids isolated in a preliminary sucrose gradient, by dialyzing them

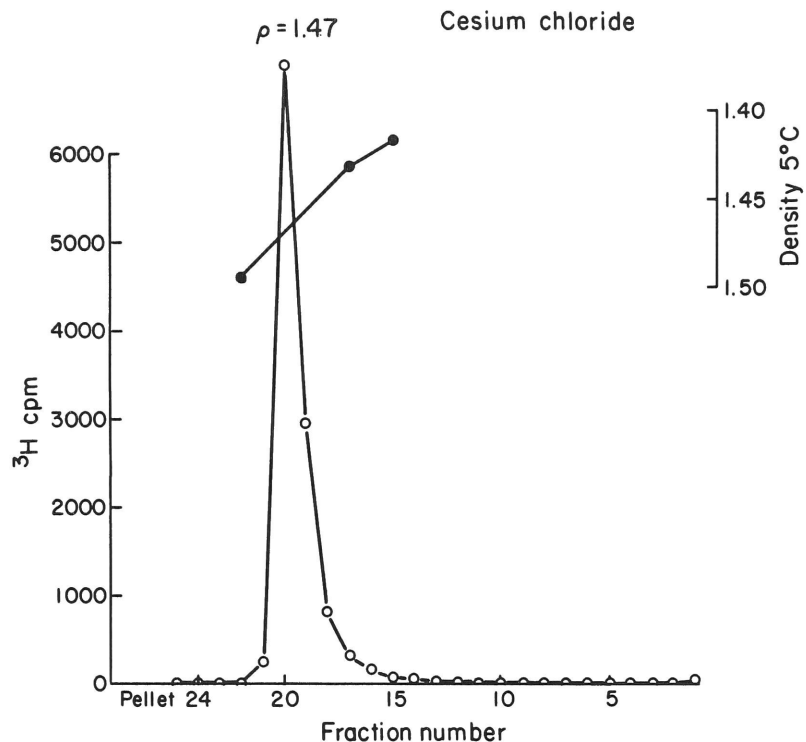


Fig. 51. Buoyant density of formaldehyde-fixed nucleocapsids in cesium chloride density gradient. One ml of ^3H -uridine labeled nucleocapsid, purified by 2 sedimentations in sucrose gradients, was fixed with 4% formaldehyde at 0 C for 1 hour, and layered over a step gradient formed from three 1.2 ml layers of cesium chloride in RSB, with densities at 5 C of 1.45, 1.40, and 1.35 gm/cc, respectively. The gradient was centrifuged in the SW-50 rotor at 100,000 x g for 28 hours at 5 C. Fifteen-drop fractions were collected from the bottom of the tube, and aliquots were assayed for acid-precipitable radioactivity (open circles). Measurements of refractive index at 25 C were made on selected fractions, and densities at 5 C (filled circles) were calculated from an empirically determined relation between refractive index and density.

against a buffer lacking Mg^{++} . The nucleocapsids are then centrifuged twice in succession into concentrated sucrose gradients of high viscosity, which both separates nucleocapsids from the dissociated ribosomal subunits and concentrates them into a narrow band.

Other authors have reported the isolation of nucleocapsids from cells infected with group A arboviruses (Friedman and Berezesky, 1968; Sreevalsan and Allen, 1968), but none has attempted to purify the nucleocapsids beyond a single sedimentation in a sucrose- RSB gradient. These preparations are probably only 5 to 20% nucleocapsid material, most of the remainder being ribosomes.

2. Chemical Composition

Semliki Forest virus nucleocapsids contain the 45 S viral RNA and two proteins. They are not disrupted by sodium deoxycholate or Triton X-100, detergents which solubilize lipids. Sreevalsan and Allen (1968) reported that radioactive choline is not incorporated into nucleocapsids of western equine encephalitis virus; Strauss et al. (1968) isolated nucleocapsids from ^{32}P -labeled Sindbis virus particles by mild deoxycholate treatment and found that all the ^{32}P in nucleocapsids was present in the RNA. Thus, the nucleocapsids of group A arboviruses probably do not contain lipids.

Although the percent composition of RNA and protein in purified nucleocapsids has not been directly measured, two indirect indices - the 260 nm/280 nm ultraviolet absorption ratio and the buoyant density in salt gradients - suggest an RNA content higher than 30%, that of most enteroviruses. Semliki Forest virus nucleocapsids are similar to eukaryotic ribosomes with regard to these parameters. For example, the glutaraldehyde-fixed 40 S ribosomal subunit of HeLa cells has a buoyant density of 1.49 gm/cc in cesium chloride (Baltimore and Huang, 1968), close to the value (1.47 gm/cc) for fixed Semliki Forest virus nucleocapsids. The 260 nm/280 nm absorbance ratio of chick embryo cell ribosomes is approximately 1.80 (unpublished observations), similar to that of nucleocapsids. Vertebrate cell ribosomes generally have RNA contents of 40 to 50% of their total mass.

3. Structure and Symmetry

Semliki Forest virus nucleocapsids are regular in size, and roughly spherical. There is no indication whatsoever of any helical structure. It is thus most probable that they are isometric bodies constructed from identical protein subunits arranged with icosahedral symmetry, the thermodynamically most probable arrangement of subunits in a closed shell (Caspar and Klug, 1962). The inability to see any clear indication of symmetry in electron micrographs of the negatively-stained particles may be due to two causes. The capsid structure is labile, and although fixation stabilizes it, perhaps there is still some derangement which occurs during negative staining and drying on the grid. Alternatively, the subunits may be arranged in a manner such that the icosahedral symmetry is not immediately obvious. This is the case for poliovirus, whose subunits are probably grouped as trimers on the surface of the capsid, rather than as the more easily discerned hexamers and pentamers found on many other isometric virus capsids. The knowledge that poliovirus has icosahedral symmetry is based on X-ray diffraction evidence rather than electron microscopic examination of the external morphology of the capsid. It may be that proof of the symmetry of arbovirus nucleocapsids will have to await X-ray diffraction evidence.

If the nucleocapsid is constructed with icosahedral symmetry, then it must be made from $60T$ identical subunits, where T , the triangulation number, can take values of 1, 3, 4, 7, 9, and certain higher values (Caspar and Klug, 1962). These structural subunits, in turn, can be formed from one or several polypeptide chains. From estimates of the molecular weights of the viral proteins and the complete virus particle, it was calculated (see Appendix) that each nucleocapsid may contain approximately 280 molecules of protein 1 and 20 of protein 3. There is no obvious way in which protein 3 could fit into an icosahedral structure, for the minimum number of identical subunits must be 60. This suggests that protein 3 may have an enzymatic rather than a structural role, or may perhaps be an internal protein involved in the folding up of the RNA chain.

However, there are enough molecules of protein 1 to make a capsid whose triangulation number is 1, 3, or 4 having 60, 120, or 240 identical subunits. These subunits would be made of, respectively, 4, 2, or 1 molecules of protein 1, utilizing in each case a total of 240 polypeptide chains in the construction of the capsid. The estimates made in the calculations are probably good enough to exclude the next higher triangulation number, 7, which would require 420 subunits. More precise molecular weight data on the individual proteins and the virus particle, and more detailed morphological studies of the nucleocapsid will be required to define more clearly its structure and symmetry. It should be pointed out that the arboviruses are unique among the RNA viruses in having an isometric capsid enclosed within an envelope.

5. RNase Sensitivity

The accessibility of the RNA within the nucleocapsid of Semliki Forest virus to ribonuclease digestion distinguishes it from most other animal virus nucleocapsids, which are ribonuclease-resistant. The protection of the viral nucleic acid from enzymatic attack is thought to be one of the major functions of the capsid protein. However, in the case of an enveloped virus, the envelope may conceivably serve as an adequate barrier to nucleases, rendering the protective function of the capsid protein superfluous. That seems to be the case for group A arboviruses. Pfefferkorn et al (1967) have shown that intact Sindbis virus particles are resistant to ribonuclease attack, although the nucleocapsids of three group A arboviruses (Sindbis: Strauss et al., 1968; western equine encephalitis: Sreevalsan and Allen, 1968; Semliki Forest: this report) are ribonuclease sensitive. However, the helical nucleocapsid of SV5, an enveloped paramyxovirus containing single-stranded RNA, is highly resistant to ribonuclease attack (Compans, 1968). Thus the possession of an envelope is not necessarily associated with ribonuclease sensitivity of the nucleocapsid. As Caspar (1965) has pointed out, the RNA in a helical nucleocapsid can be bonded along its entire length to protein subunits. Thus, a helical nucleocapsid can protect its RNA from nuclease attack simply by covering all of the RNA with protein, but this is topologically impossible for a linear nucleic acid molecule in an isometric capsid. Nuclease resistance in icosahedral nucleocapsids

is probably achieved by creating a barrier to the penetration of nucleases into the interior of the nucleocapsid. This concept derives support from experiments on noninfectious poliovirus particles produced in the presence of the amino acid analogue, p-fluorophenylalanine (FPA) (Hummeler and Wecker, 1964). These authors showed that the abnormal virus particles so produced contain infectious RNA which can be inactivated in situ by ribonuclease treatment, although the RNA within normal particles is completely protected from ribonuclease attack. Negative stain (phosphotungstate) penetrates the abnormal particles, while it is excluded from normal poliovirus particles. The authors concluded that a structural change in the protein subunits, brought about by the substitution of FPA for phenylalanine, leads to the production of virus particles which are more permeable both to ribonuclease and to phosphotungstate. Similar conclusions were reached concerning the ribonuclease sensitivity of mutant particles of the bacteriophage R17 (Argetsinger and Gussin, 1966), and altered particles of turnip yellow mosaic virus treated with an alkylating agent (Kaper and Jenifer, 1968).

Several RNA-containing plant viruses, including cucumber mosaic virus (CMV), cowpea chlorotic mottle virus (CCMV), and alfalfa mosaic virus (AMV), have been shown to be ribonuclease sensitive in their naturally-occurring forms (Francki, 1968; Bancroft et al., 1967; Pirone, 1962). Fragments of RNA are released from AMV during incubation with ribonuclease (Bol and Veldstra, 1969). AMV particles treated with ribonuclease aggregate in low ionic strength buffer, or are degraded into smaller particles in higher ionic strength buffer, but remain intact even after losing 80-100% of their RNA if fixed with formaldehyde before treatment (Bol and Veldstra, 1968).

There are several properties in addition to ribonuclease-sensitivity, shared by these viruses and the nucleocapsids of Semliki Forest virus, which suggest that they may be structurally similar. Both AMV and CCMV are broken down in high salt (NaCl or CsCl) (Hull et al., 1969; Bancroft et al., 1968), but can be stabilized by formaldehyde fixation. AMV and CMV also break down in 1% phosphotungstate (Gibbs et al., 1963; Francki et al., 1966) unless prefixed. Semliki Forest virus nucleocapsids

apparently disintegrate in CsCl (~ 3.7 M), and have an irregular appearance indicating partial disruption when negatively-stained with phosphotungstate unless prefixed with formaldehyde. Bancroft et al. (1967) suggested, on the basis of the sensitivity of the capsid of CCMV to high salt, that the protein subunits of CCMV are held in place mainly by ionic bonds between the viral RNA and the subunits, and that there is little bonding between the subunits themselves. The same may be true for Semliki Forest virus nucleocapsids.

AMV was found to absorb up to 20% of its weight of cytochrome c, a basic protein (Hull et al., 1969). Fixed particles from which the RNA has been removed by ribonuclease treatment no longer absorb cytochrome c. Therefore, it was suggested that the basic protein is adsorbed to the viral RNA within the virus particle. This kind of phenomenon may explain the finding that 25% of the protein in purified Semliki Forest virus nucleocapsid preparations is of cellular rather than viral origin. If the RNA within nucleocapsids is exposed, either because of a loose capsid structure or because it is partly on the surface of the particle, it may adsorb basic proteins present in cell extracts during isolation. These proteins may remain adsorbed in the ionic conditions (0.1 M salt) under which the nucleocapsids are purified. The ribonuclease sensitivity of the RNA in situ may be another manifestation of the exposed position of the RNA.

The Number of Molecules of the Three Viral Proteins per Virus Particle

The number of molecules of proteins 1, 2, and 3 per virus particle can be calculated if the following data are known: (1) the molecular weight of each protein; (2) the proportion of the total virus protein represented by each protein; (3) the total mass of protein per virus particle. Approximate molecular weights of the three viral proteins (1: 35,000; 2: 55,000; 3: 70,000 daltons) are estimated in section 7 of Part III of this thesis. The relative proportions of radioactivity (1:2:3 = 1:4:0.15) in the three protein peaks on acrylamide gels should reflect their respective mass contributions to the viral protein, because the individual proteins should have been labeled equivalently by the mixture of 13 amino acids in which nucleocapsids and virus were grown.

The total mass of protein per virus particle can be calculated from the percent composition of RNA and protein in the virus, and the molecular weight of the RNA. Neither of these quantities is known for Semliki Forest virus, but the composition of the closely related Sindbis virus (mass ratio of protein to RNA = 11:1) is known from the work of Pfeifferkorn and Hunter (1963). The molecular weight of the RNA of Sindbis virus has been reported to be 4 to 5 million daltons (Burge et al., 1969) although the data have not been published. Use of the Spirin (1961) formula ($M \text{ Wt} = 1550 S^{2.1}$), derived empirically from independent data on the molecular weights of a number of different RNA's, leads to a similar value of 4.6 million for an RNA with an $S_{20,w}$ of 45. Taking a value of 4.5 million for the estimated molecular weight of the RNA, and assuming there is only one RNA strand per virus particle, the total protein per virus particle is $11 \times 4.5 = 50$ million daltons.

Table V shows that, using the above values, it can be calculated that there are approximately 280 molecules of protein 1 (the major nucleocapsid protein), 750 of protein 2 (the envelope protein), and 20 of protein 3 (the minor nucleocapsid protein) per virus particle.

TABLE V

Estimation of the Number of Protein Molecules
of Each Type Per Virus Particle

Protein	Proportion of total virus protein	Total mass per virus particle	Molecular weight	Number of molecules per virus particle
1	1 part	9.7×10^6	35,000	277
2	4 parts	39×10^6	55,000	709
3	0.15 part	1.5×10^6	70,000	21

Using the same parameters the molecular weight of the nucleocapsid may be estimated. Assuming the nucleocapsid contains only the viral RNA and proteins 1 and 3, its molecular weight is the sum of their individual molecular weights, i.e. $(4.5 + 9.7 + 1.5) \times 10^6 = 15.7 \times 10^6$ daltons. It should be emphasized that this value is only very approximate, for it depends upon a number of assumptions outlined above. A comparison of the sedimentation coefficient of Semliki Forest virus nucleocapsids (145 S) with that of poliovirus (160 S), which has a molecular weight of 7 million daltons, suggests that the value of 15.7 million daltons is an over-estimate.

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