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Replication of Sendai Virus

II. Steps in Virus Assembly

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Chick embryo fibroblast cultures infected with Sendai virus were incubated with ^{3}H-uridine in the presence of actinomycin D beginning at 18 hr after infection. The 35 and 18*S* virus-specific ribonucleic acid (RNA) components were found in a ribonuclease-sensitive form in the cell and appeared to be associated with polyribosomes. Newly synthesized 57*S* viral RNA was rapidly coated with protein to form intracellular viral nucleocapsid, and no 57*S* RNA was found "free" (ribonuclease-sensitive) in the 2,000 × *g* supernatant fraction of disrupted cells. The nucleocapsid from detergent-disrupted Sendai virus and that from disrupted cells were indistinguishable in ultrastructure and buoyant density, and neither was found to be infectious or have hemagglutinating activity. Kinetic studies of nucleocapsid and virus formation indicated a relative block in conversion of viral nucleocapsid to complete enveloped virus in these cells, resulting in accumulation of large amounts of nucleocapsid in the cell cytoplasm.

In a previous paper (2), we showed that several distinct components of virus-specific ribonucleic acid (RNA) are synthesized in chick embryo fibroblast cultures infected with Sendai virus, a subgroup 2 myxovirus. One component, representing about one-fourth of the newly synthesized RNA, appears to be identical to the 57*S* RNA recovered from whole virus. The remaining virus-specific RNA consists of three distinct components sedimenting at 35, 22, and 18*S*, and 90% or more of this RNA appears to be complementary in base sequence to viral RNA, as indicated by its annealing to viral RNA. Previous studies with a similar subgroup 2 myxovirus, Newcastle disease virus (NDV), indicated that virus-specific RNA was synthesized in the cell cytoplasm and that much of the complementary RNA was associated with polyribosomes of the cell (3). The state of the 57*S* viral RNA after its synthesis in cells infected with Sendai virus or NDV is not known, although some or all of it may enter the viral nucleocapsid, as was demonstrated in the cytoplasm of cells infected with several subgroup 2 myxoviruses (5–7, 10, 13), and some or all may eventually enter whole virus which is formed at the cell surface (7, 10).

In this study, we attempted to determine the location and state of the newly synthesized RNA components within Sendai virus-infected chick embryo cells and to study the incorporation of viral RNA into whole virus. No 57*S* viral RNA was found free in the cell cytoplasm in these experiments. Instead, the 57*S* RNA appeared to be immediately coated with protein after its synthesis, making it insusceptible to digestion by ribonuclease. It was quantitatively recovered from the cell cytoplasm as part of the helical viral nucleocapsid. The smaller RNA components were found in the cell cytoplasm in a ribonuclease-susceptible form, and they sedimented in the region of polyribosomes and single ribosomes. Kinetic studies showed that ^{3}H-uridine first appears in the 57*S* RNA of the nucleocapsid recovered from the cell cytoplasm; second, in nucleocapsid released from cells with trypsin; third, in whole virus released from cells with trypsin; and finally in whole virus in the culture medium. Quantitation of the 57*S* RNA in these pools indicates that more than 95% of all of the newly synthesized 57*S* RNA remains in the form of nucleocapsid in the cell cytoplasm, and that less than 5% enters whole virus.

MATERIALS AND METHODS

Materials. Uridine-5-^{3}*H* (25 c/mmole) and carrier-free $H_3{}^{32}PO_4$ were purchased from New England Nuclear Corp., Boston, Mass. Bovine pancreatic ribonuclease was purchased from Worthington Biochemical Corp. Trypsin was purchased from Difco

[1] Part of this work was taken from a thesis submitted by C. D. B. in partial satisfaction of the requirements for the Doctor of Philosophy degree from the University of California, Berkeley.

and was prepared as a 0.25% solution (w/v) in Tris-(hydroxymethyl)aminomethane(Tris)-buffered saline. *Vibrio cholerae* neuraminidase (500 units/ml) was purchased from Calbiochem, Los Angeles, Calif. Tween 80 (polyoxyethylene sorbitan-mono-oleate) was purchased from J. T. Baker Chemical Co. Actinomycin D was the gift of Merck & Co., Inc., Rahway, N.J. Nonidet 40 (NP40) was the gift of Shell Chemical Co. Tobacco mosaic virus (TMV) was the gift of C. A. Knight.

Standard buffer. The buffer contained 0.1 M NaCl, 0.01 M Tris-hydrochloride (*p*H 7.4), and 0.001 M ethylenediaminetetraacetic acid (EDTA).

Viruses. The Harris strain of Sendai virus and the L-Kansas 48 strain of NDV were grown in embryonated eggs, and the chorioallantoic fluid containing 10^8 to 10^9 egg infectious units/ml was used to infect cells in tissue culture (2).

Cell cultures. Secondary cultures of chick embryo fibroblasts were cultured and infected with virus as previously described (2).

Assays for infectivity and hemagglutination. Sendai virus infectivity was measured by an end point dilution method in embryonated eggs as previously described (2).

Quantitative assay for hemagglutination was done by preparing serial twofold dilutions of the fluid to be tested in phosphate-buffered saline (PBS), mixing with chicken erythrocytes, and incubating as previously described (2).

Virus purification. Virus was purified from tissue culture medium or chorioallantoic fluid by differential centrifugation and sedimentation in sucrose gradients as previously described (2, 3).

Isolation of nucleic acids. Nucleic acid from cells or virus was isolated by the use of sodium dodecyl sulfate (SDS) and phenol (3). When the amount of nucleic acid in the sample to be extracted was very small, 2 mg of TMV carrier was added before phenol extraction.

Preparation of cytoplasmic extracts. Two methods were used to disrupt chick embryo cells. One method has been previously described (3). Cells growing in a monolayer were removed by addition of 0.25% trypsin and incubated for 5 min at room temperature with occasional agitation. Suspended cells were washed with PBS, suspended in ice-cold hypotonic buffer containing 0.01 M NaCl, 0.01 M Tris-hydrochloride (*p*H 7.4), and 0.002 M $MgCl_2$, and allowed to swell for 5 min. Cells were broken by 15 to 16 strokes of a tight-fitting Dounce homogenizer (Kontes Glass), leaving most nuclei intact. The broken-cell suspension was centrifuged at 4,000 rev/min for 5 min in the Sorvall SS34 rotor to remove unbroken cells, nuclei, and large membrane fragments. The supernatant fraction was used as the cytoplasmic extract. A second method of cell disruption used the detergent NP-40 (16). The medium of cell cultures was replaced with 1 to 2 ml of 0.5% NP40 in 0.1 M NaCl, 0.01 M Tris-hydrochloride, and 0.002 M $MgCl_2$. After 5 min of incubation with gentle agitation at room temperature, cell fragments remaining on the dish were scraped off with a rubber policeman. Nuclei, which remained intact, and large cell fragments were removed by centrifugation at 4,000 rev/min for 5 min, and the supernatant fluid was used as the cytoplasmic extract.

Electron microscopy. Infected cells were fixed as a monolayer in 1.6% glutaraldehyde in PBS (*p*H 7.2) with 0.005 M $MgCl_2$ and 0.0001 M $CaCl_2$ (PBS MgCa). The cells were then scraped off the dish, centrifuged to a pellet in McNaught protein tubes, and fixed as a pellet in 1% osmic acid in PBS MgCa. After dehydration in a graded ethyl alcohol-0.15 M NaCl series, infiltration with propylene dioxide, and embedding in Epon 812, thin sections were cut on an LKB ultramicrotome. Contrast was increased by staining with 1% aqueous uranyl acetate and 1% alkaline lead citrate (14).

Virus or nucleocapsid was pelleted in the Spinco SW50 rotor by spinning at 47,000 rev/min for 90 min. The pellet was resuspended in a small volume of distilled water, and a small drop of this suspension was delivered to a collodion-covered 200-mesh copper grid. Negative stain was 2% phosphotungstic acid adjusted to *p*H 7 with KOH.

Micrographs were taken on a Siemens Elmiskop I electron microscope at either 60 or 80 kv with a 50-μm objective aperture.

Scintillation counting. Radioactivity precipitated in 5% trichloroacetic acid was collected and washed on Millipore filters and counted in toluene scintillation fluid in a Packard Tri-Carb scintillation spectrometer (3).

RESULTS AND DISCUSSION

Fractionation of cytoplasmic extracts from Sendai virus-infected cells. Chick embryo fibroblast cultures were incubated with ^{3}H-uridine in the presence of actinomycin D from 18 to 20 hr after infection with Sendai virus, when virus-specific RNA synthesis is maximum (2). Under these conditions, all labeled RNA has been shown to be virus-specific RNA (2). A cytoplasmic extract was then prepared by using NP40 detergent and divided into three parts for sedimentation on sucrose density gradients. Figure 1A shows the distribution in a sucrose gradient of trichloroacetic acid-precipitable radioactivity and cytoplasmic material absorbing at 260 nm after sedimentation of a sample of the cytoplasmic extract layered directly on a sucrose gradient. The A_{260} peak in fraction 23 represents single ribosomes (74*S*), and that in fraction 27, the 50*S* ribosomal subunit. The ultraviolet-absorbing material sedimenting more rapidly than single ribosomes (fractions 1 to 20) represents polyribosomes. A significant fraction of the radioactive RNA sediments in a broad distribution with polyribosomes and the single ribosomes. A distinct radioactive component with a peak in fraction 8 sediments more rapidly than the single ribosomes and most of the polyribosomes detected by ultraviolet absorbancy. Some radioactive material sediments more slowly than single ribosomes.

Figure 1B shows the results of sedimentation of a second sample of cytoplasmic extract after incu-

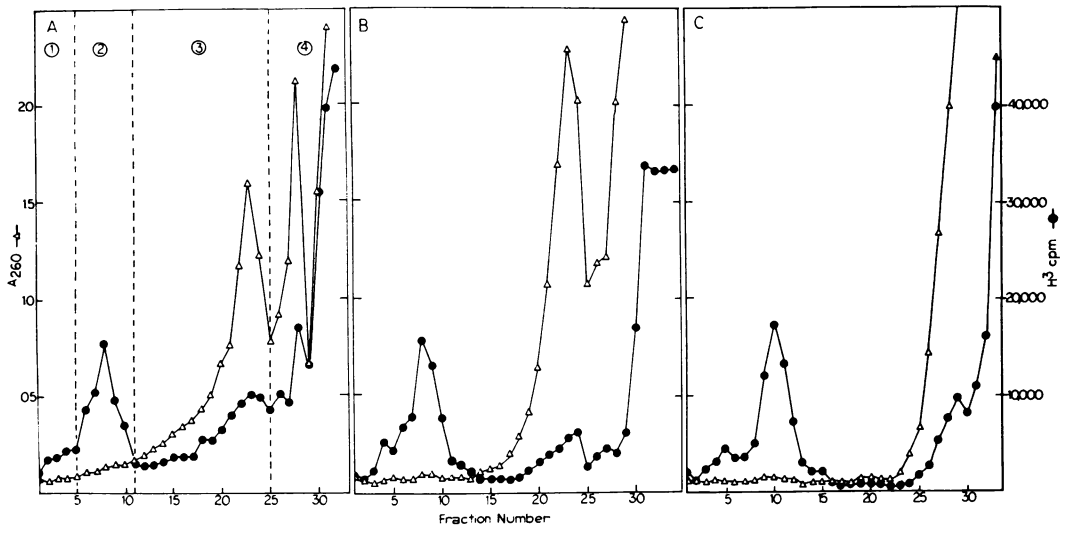

FIG. 1. *Sedimentation of cell extracts from Sendai virus-infected cells. Four 150-mm tissue culture dishes with 2 × 10^7 cells each were infected with Sendai virus. Eighteen hours later, the culture medium of each was replaced with 10 ml of medium containing 2 μg of actinomycin D per ml; 30 min later, 300 μc of ^{3}H-uridine was added to each culture. After 2 hr, the culture medium was removed, and a cytoplasmic extract was prepared from the cells of all four cultures by using 0.5% NP40. The 4,000 rev/min supernatant fraction was divided into three parts. The first portion (A) was layered directly onto a gradient of 5 to 30% sucrose in 0.1 M NaCl, 0.01 M Tris (pH 7.4), and 0.002 M $MgCl_2$. The second portion (B) was incubated with 5 μg of pancreatic ribonuclease for 5 min at room temperature before layering on a second sucrose gradient. EDTA was added to a third portion (C) to make a final concentration of 0.004 M before layering the extract on a third sucrose gradient. All three sucrose gradients were centrifuged for 2.5 hr at 25,000 rev/min and 4 C in a Spinco SW 25.3 rotor and analyzed for trichloroacetic acid-precipitable radioactivity and A_{260} as previously described (2).*

bation with pancreatic ribonuclease. The increase in ultraviolet-absorbing material in the single-ribosome peak (around fraction 23) and the decrease in polyribosomes, indicated by the decrease in absorbancy around fraction 15 (*compare* Fig. 1A), resulted from ribonuclease action on polyribosomes. It can be seen that ribonuclease did not alter the sedimentation behavior of the rapidly sedimenting radioactive component around fraction 8 or the amount of trichloroacetic acid-precipitable radioactivity in this component.

Figure 1C shows the results of sedimentation of a third portion of cytoplasmic extract to which EDTA had been added. The reduction in the amount of ultraviolet-absorbing material in fractions 1 to 23 is consistent with dissociation by EDTA of polyribosomes into more slowly sedimenting components. It also appears that EDTA did not alter the sedimentation behavior or the amount of trichloroacetic acid-precipitable radioactivity in the rapidly sedimenting component around fractions 9 and 10. The amount of this RNA-containing component appears to be too small to be detected by absorbancy at 260 nm.

These results suggest that some newly synthesized RNA in Sendai virus-infected cells sediments with polyribosomes, single ribosomes, and a significant fraction of new RNA in a rapidly sedimenting structure. The sedimentation behavior of this structure is not altered by ribonuclease or EDTA, and the labeled RNA associated with it is not made trichloroacetic acid-soluble by ribonuclease. In experiments in which infected cells were disrupted by a Dounce homogenizer without NP40 detergent, as previously described, the sucrose gradient profiles and the amount of radioactive RNA in each component in the gradients were the same as in the experiment shown in Fig. 1, indicating that the rapidly sedimenting structure does not result from the action of NP40 on infected cells or whole virus associated with the cells.

Identification of RNA in cytoplasmic extracts. To identify the RNA in different regions of the sucrose gradient shown in Fig. 1A, the fractions in each of the regions designated 1 to 4 were pooled, and the RNA was recovered from each by phenol extraction by using 50 μg of TMV RNA as carrier. The purified RNA from each region was then fractionated by sucrose gradient sedimentation (Fig. 2).

Figure 2A shows the results with the RNA from region 1 in Fig. 1A. Sedimentation was from right to left. The component of RNA detected by

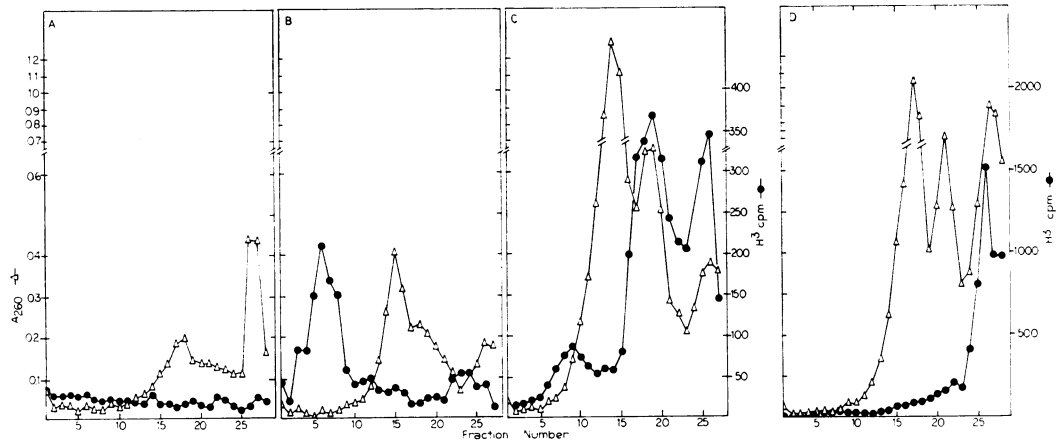

FIG. 2. *Sedimentation of RNA extracted from four regions of the sucrose gradient shown in Fig. 1A. All fractions in each of the numbered regions of the sucrose gradient in Fig. 1A were pooled, 2 mg of TMV was added as carrier, and RNA was extracted from each. Each RNA sample was then sedimented in a sucrose density gradient in a Spinco SW 50 rotor at 47,000 rev/min and 4 C for 105 min as previously described* (2).

A_{260} with a peak in fraction 18 (Fig. 2A) is the small amount of TMV RNA used as carrier. No distinct component of radioactive RNA is apparent.

The RNA from region 2 in Fig. 1A, which includes the rapidly sedimenting radioactive structure, is shown in Fig. 2B. The RNA component detected by A_{260} with a peak in fraction 15 (Fig. 2B) represents TMV RNA and a small amount of 28*S* ribosomal RNA which sediments with TMV RNA under these conditions. Almost all of the radioactive RNA sediments like 57*S* viral RNA, and no significant amount of radioactive 18, 22, or 35*S* virus-specific RNA is present.

The results of sedimentation of the RNA from region 3 in Fig. 1A, which includes most of the polyribosomes and single ribosomes detected by ultraviolet absorbancy, are shown in Fig. 2C. The RNA components detected by A_{260}, with peaks in fractions 14 and 19 (Fig. 2C), consist mostly of 28 and 18*S* ribosomal RNA, respectively. Two of the components of radioactive RNA sediment like the 35*S* (fraction 9) and the 18*S* (fraction 19) virus-specific RNA forms, which have been shown to be almost completely complementary in base sequence to viral RNA (2). Some radioactive RNA (fraction 25) sediments more slowly than 18*S* and may represent virus-specific RNA degraded during the preparation of the cytoplasmic extract. No labeled 57*S* RNA was recovered from this region of the gradient (e.g., region 3, Fig. 1A) in several experiments.

The RNA from region 4 in Fig. 1A, which includes material sedimenting more slowly than single ribosomes, is shown in Fig. 2D. Again, ribosomal RNA is detected by A_{260}, and almost all of the radioactive RNA (fraction 25) sediments more slowly than 18*S*.

These results suggest that all newly synthesized 57*S* virus-specific RNA from Sendai virus-infected cells can be recovered from the rapidly sedimenting ribonuclease-resistant structure (Fig. 1), and the 35 and 18*S* virus-specific RNA forms sediment in a ribonuclease-sensitive form with polyribosomes and single ribosomes. The latter finding is in agreement with experiments with NDV-infected cells, in which the 18 and 35*S* virus-specific RNA components sedimented with polyribosomes (3).

Identification of the rapidly sedimenting ribonuclease-resistant structure in cytoplasmic extracts as viral nucleocapsid. A number of the properties of the rapidly sedimenting ribonuclease-resistant structure from infected cells (Fig. 1) indicate that it is a viral nucleocapsid. Consistent with this are the observations that its sedimentation was not altered by ribonuclease (Fig. 1B) or EDTA (Fig. 1C), that the radioactive RNA associated with the structure was not made trichloroacetic acid-soluble by ribonuclease (Fig. 1B), and that RNA with sedimentation characteristics of viral RNA (57*S*) was exclusively recovered from this component in many experiments. Kingsbury and Darlington (12) have shown that the nucleocapsid recovered from detergent-disrupted NDV contains high molecular weight RNA, and that ribonuclease does not alter the sedimentation rate of the nucleocapsid or degrade the RNA to a trichloroacetic acid-soluble form.

The buoyant density of the rapidly sedimenting structure from Sendai virus-infected cells, determined by equilibrium centrifugation in a preformed sucrose density gradient, was 1.26 to 1.27 g/ml. (Fig. 3). This value is significantly greater than the buoyant density value of whole virus, which is 1.22 g/ml under the same conditions. Compans and Choppin (5) found a buoyant density of 1.30 g/ml in a CsCl density gradient for the nucleocapsid isolated from SV-5-infected BHK cells.

Figure 4 shows the results of rate zonal sedimentation of the rapidly sedimenting component (closed circles) in a 5 to 20% sucrose density gradient. TMV (open triangles), which has a sedimentation coefficient of about 160 $S_{20,w}$ under these conditions, was used as a sedimentation marker in the experiment. The radioactive cytoplasmic component clearly sediments more rapidly than TMV and can be estimated to have a sedimentation coefficient of approximately 200S under these conditions. The sedimentation coefficient of intact NDV, a virus with almost identical size and shape as Sendai virus, has been estimated to be 1,100S (15). Thus, whole virus has a much greater rate of sedimentation than that of the cytoplasmic component in Fig. 4.

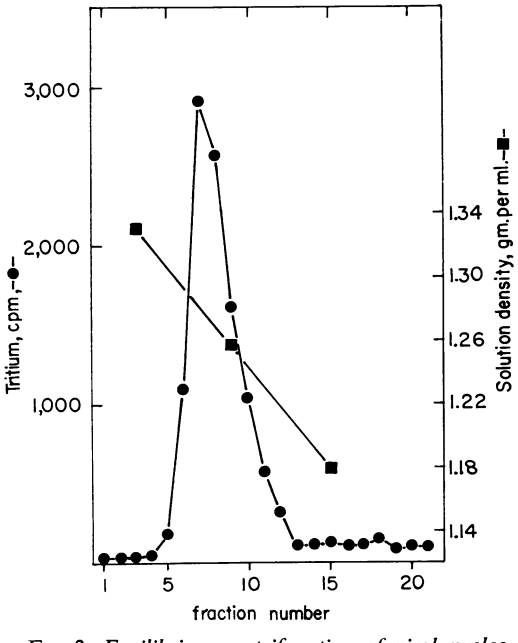

FIG. 3. *Equilibrium centrifugation of viral nucleocapsid. ^{3}H-labeled nucleocapsid, prepared as described in the legend to Fig. 1C, was layered over a preformed 15 to 65% sucrose gradient containing standard buffer and centrifuged at 47,000 rev/min for 4 hr at 4 C in a Spinco SW 50 rotor.*

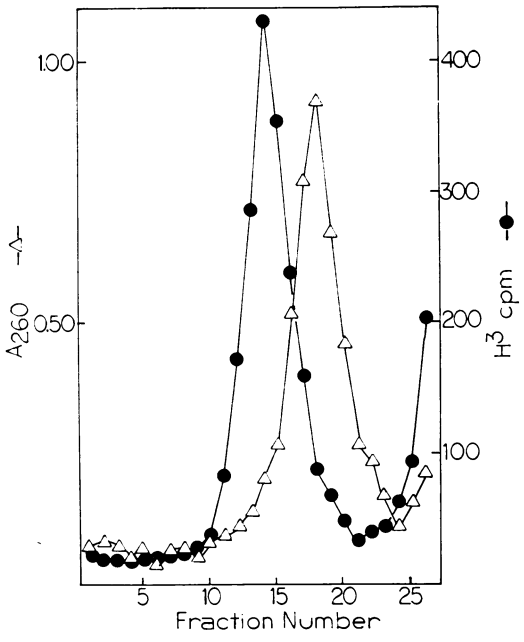

FIG. 4. *Velocity sedimentation of nucleocapsid. ^{3}H-labeled nucleocapsid, prepared as described in Fig. 1C, was mixed with unlabeled TMV, and the mixture was layered over a 5 to 20% sucrose density gradient and centrifuged at 45,000 rev/min and 4 C for 15 min in an SW 50 rotor.*

Kingsbury and Darlington (12) have estimated the sedimentation coefficient of the nucleocapsid from detergent-disrupted NDV to be 200S.

To determine the morphological appearance of the 200S cytoplasmic structure, it was examined by electron microscopy after negative staining. Whole virus grown in the chorioallantoic cavity was also examined for comparison. Figure 5A shows that typical viral nucleocapsid is present in the sucrose gradient in the position of the 200S structure. The nucleocapsid is indistinguishable from that described in detail from SV-5-(5) and NDV-(6) infected cells and from disrupted Sendai virus (4, 9) and NDV (12).

Figure 5B shows whole Sendai virus. The nucleocapsid within virions and that lying outside have the same morphology as the cytoplasmic cleocapsid in Fig. 5A.

Comparison of cytoplasmic nucleocapsid with whole virus and detergent-disrupted virus. Further evidence that the rapidly sedimenting cytoplasmic structure is viral nucleocapsid was obtained by testing its infectivity and hemagglutinating activity (HA) and by comparing it with whole virus and detergent-disrupted virus. Figure 6A shows the results of equilibrium sedimentation of $^{32}PO_4$-labeled Sendai virus after disruption with 0.3%

sodium deoxycholate (DOC) and layering over a preformed sucrose density gradient. The heterogeneous ^{32}P-labeled component with a peak in fraction 6 has the buoyant density of the nucleocapsid isolated from cells (1.27 g/ml). It was demonstrated that almost all of the ^{32}P in this component after phenol extraction was in the form of 57*S* RNA, which is consistent with its being viral

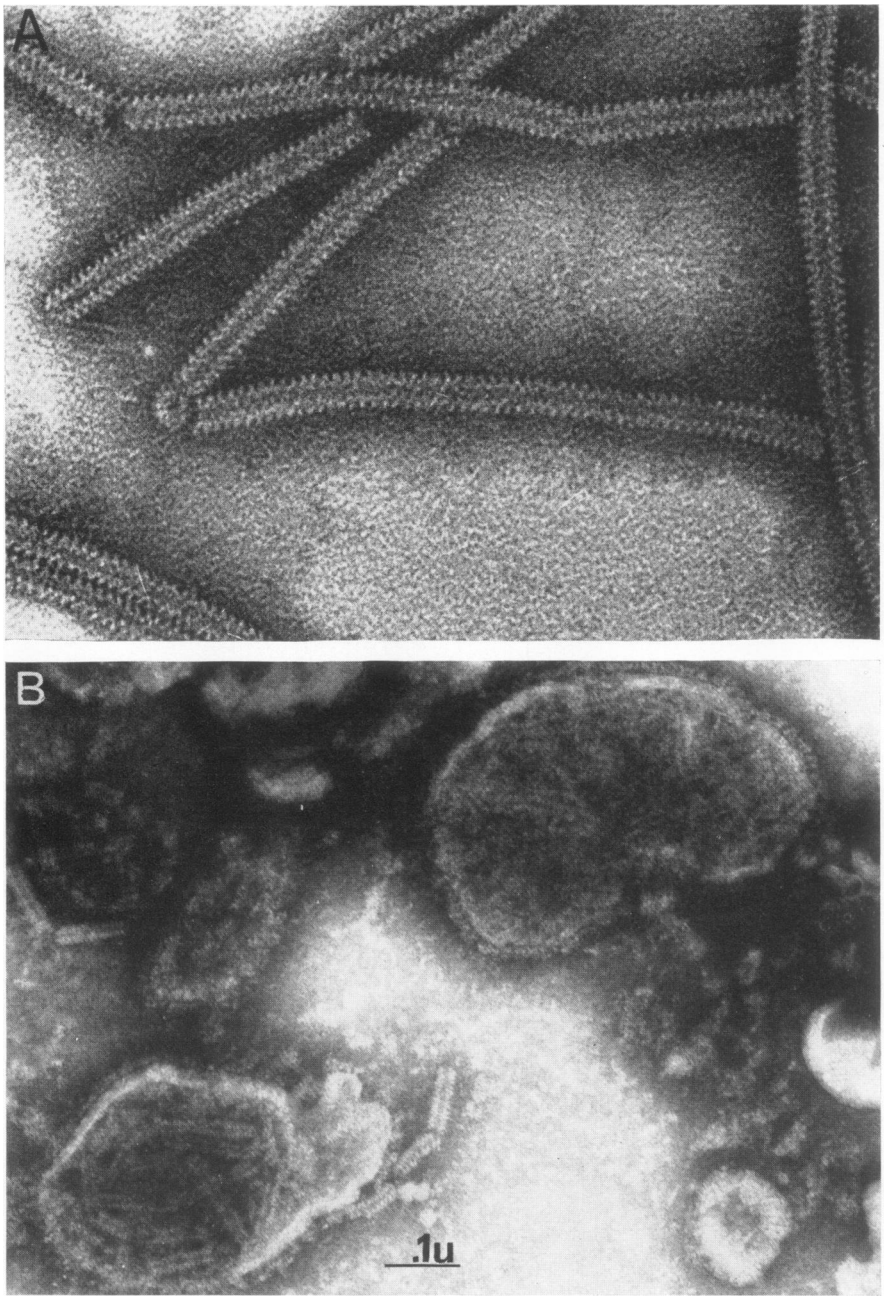

FIG. 5. *Morphology of cellular nucleocapsid and virus. (A) Nucleocapsid was prepared from infected cells as described in Fig. 1, and sucrose gradient fractions containing ^{3}H-nucleocapsid were centrifuged to make a pellet for electron microscopy as described in the text. × 240,000. (B) Sendai virus grown in embryonated eggs and purified in sucrose gradients was prepared for electron microscopy as described in the text. × 100,000.*

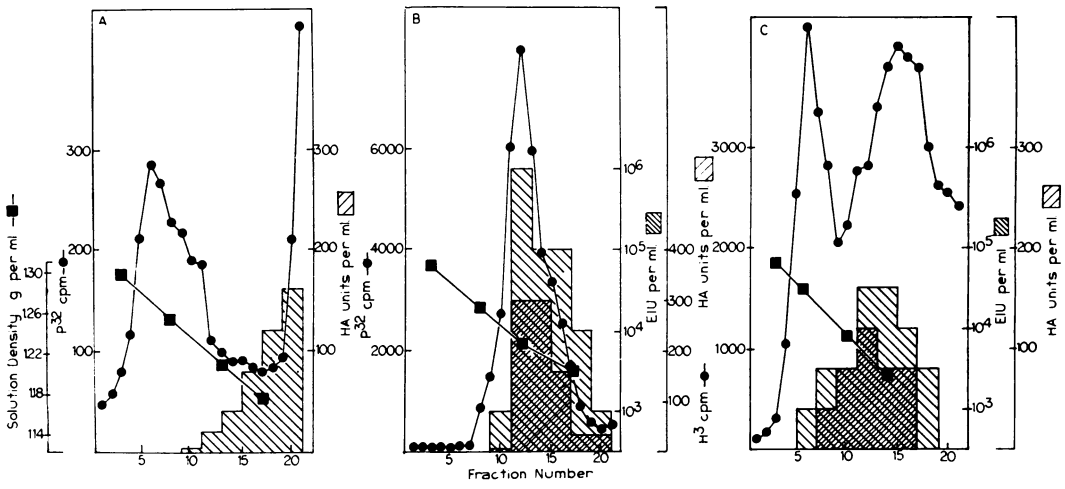

FIG. 6. *Comparison of nucleocapsid from cells, nucleocapsid from virus, and intact virus. Sendai virus-labeled with $^{32}PO_4$ was prepared in embryonated eggs and purified as previously described (2). To one sample of $^{32}PO_4$ virus (A), DOC was added to a final concentration of 0.3%, and the mixture was shaken for 5 min at room temperature and then layered over a 15 to 65% sucrose gradient. A second sample of $^{32}PO_4$ virus (B) was layered directly on an identical sucrose gradient. A cytoplasmic extract was prepared from seven infected 150-mm cultures after incubation with ^{3}H-uridine and actinomycin D as described in Fig. 1, and the extract (C) was layered over a third 15 to 65% sucrose density gradient.*

All three samples were centrifuged for 14 hr at 25,000 rev/min and 4 C in a Spinco SW 25.3 rotor. Fractions were collected dropwise from the bottom of the tube, and trichloroacetic acid-precipitable radioactivity was determined for 0.1-ml samples. A 0.05-ml amount was removed from each fraction, diluted in serum-free 199A, and frozen for later infectivity and HA assays as described in the text. Densities were determined by weighing 100-μliter portions in a micropipette.

nucleocapsid released from DOC-treated virus. No viral infectivity was associated with DOC-treated virus, and HA was only associated with material sedimenting near the top of the gradient (fractions 10 to 21). Figure 6B shows the results of sedimentation of ^{32}P-labeled whole virus before treatment with DOC and demonstrates that radioactivity, infectivity, and HA coincide at a density of 1.23 g/ml (around fraction 12). Figure 6C shows the results of equilibrium sedimentation of a cytoplasmic extract from infected cells prepared as described for the experiment in Fig. 1. The tritium-labeled component with a peak in fraction 6 has the buoyant density of nucleocapsid (1.27 g/ml). The material with HA has a peak with a density around 1.22 to 1.23 g/ml and represents whole virus. Infectivity and HA do not appear to be specifically associated with the nucleocapsid in this experiment, and purified nucleocapsid in other experiments failed to show infectivity or HA.

These experiments suggest that nucleocapsids from detergent-disrupted Sendai virus and from the cytoplasm of infected cells are similar in buoyant density, contain 57*S* RNA, and do not have infectivity or HA. Although Bukrinskaya et al. (4) have reported infectivity associated with nucleocapsid from Sendai virus-infected cells, we have been unable to demonstrate infectivity with our nucleocapsid preparations.

Formation of nucleocapsid. To determine the time required for nucleocapsid assembly after viral RNA synthesis, an experiment was done in which the rate of ^{3}H-uridine incorporation into total 57*S* RNA and into intracellular nucleocapsid was measured, starting at 18 hr after infection. Different cultures of infected cells were incubated with high specific activity ^{3}H-uridine for 5, 10, and 15 min in the presence of actinomycin D, and the amounts of radioactivity in total 57*S* RNA of the cells and the radioactivity in intracellular nucleocapsid were determined for each incubation time. It was shown earlier (Fig. 2B) that all of the radioactive RNA extracted from the nucleocapsid is 57*S* RNA.

The results in Table 1 show that, after only 5 min of incubation with ^{3}H-uridine, all of the labeled 57*S* RNA in the cell cytoplasm can be accounted for by radioactivity in nucleocapsid. Similarly, after 10 and 15 min of incubation with ^{3}H-uridine, essentially all of the radioactivity in 57*S* RNA can be accounted for in nucleocapsid. The differences between the amounts of radioactivity in the two fractions at all three times are 10% or less, a value which is within the experimental error of the methods used. Two conclu-

TABLE 1. *Incorporation of ^{3}H-uridine into total 57S RNA in infected cells and into viral nucleocapsid*[a]

Time of ^{3}H-uridine incubation	Counts/min of ^{3}H label in	
	57S RNA	Nucleocapsid
min		
5	822	856
10	1,828	1,724
15	8,548	7,718

[a] Six 150-mm culture dishes containing 2×10^7 cells each were infected with Sendai virus. Eighteen hours later, the medium of each culture was replaced with 10 ml of medium containing 2 μg of actinomycin D per ml. Thirty minutes later, 400 μc of ^{3}H-uridine was added to each and, at the subsequent times designated, two culture dishes were placed in an ice-water bath and the medium replaced with ice-cold phosphate-buffered solution. Cells were removed from the dishes with concentrated trypsin in the cold, and the cell suspensions were divided into two parts. From one part, RNA was extracted with sodium dodecyl sulfate and phenol, and the radioactivity in 57S RNA was determined after sucrose gradient sedimentation of the RNA (2). The second part of each cell suspension was disrupted with NP40, and the radioactivity in nucleocapsid was determined after sucrose gradient sedimentation as described for Fig. 1.

sions can be made from this experiment. First, 57S RNA becomes part of the viral nucleocapsid very rapidly after its synthesis. The time is undoubtedly significantly less than 5 min, since essentially all 57S RNA is found in nucleocapsid at 5 min. This rapid assembly of Sendai virus nucleocapsid is similar to the maturation of polio virus, which has been estimated to occur within 3 min or less of the completion of synthesis of viral RNA (1). Second, not only is 57S RNA rapidly converted to nucleocapsid after its synthesis, but all or a very large fraction of it enters viral nucleocapsid. Free 57S RNA is not detected by our methods in cell cytoplasm at this stage of the virus growth cycle. The results described in Fig. 1A and 2 are in accord with these conclusions.

The results here suggest that the smaller RNA components which appear to be associated with polyribosomes and not the 57S RNA may serve as viral messenger. It is not excluded that the 57S RNA does enter polyribosomes at an earlier stage in the virus replication cycle. It has been shown that most of newly synthesized poliovirus RNA becomes associated with polyribosomes early in the polio growth cycle, and most enters complete virions late in the cycle (1).

Virus formation at the cell surface. The final steps in myxovirus assembly are thought to take place at the cell surface by the process described as budding (7, 11). We have found that treatment of Sendai virus-infected cells with trypsin quantitatively removes cell associated virus without disrupting cells. This was shown by an experiment in which cells, after incubation with ^{3}H-uridine in the presence of 2 μg of actinomycin D per ml between 18 and 20 hr after infection with Sendai virus, were gently removed from culture dishes with trypsin solution and centrifuged, and the resulting supernatant fluid was analyzed by equilibrium centrifugation in a preformed sucrose density gradient. Figure 7 shows the distribution in the gradient of the radioactive particulate material released from the infected cells by trypsin. Two incompletely separated radioactive components appear in the gradient. One has the buoyant density of whole virus (1.23 g/ml) and is associated with infectivity and HA. The other

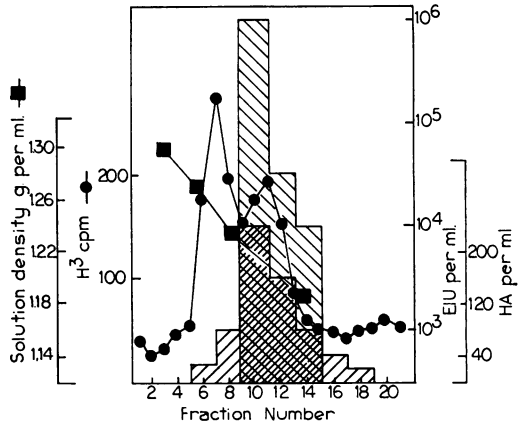

FIG. 7. *Particulate material released from cells by trypsin. Seven 150-mm cultures of Sendai virus-infected cells were incubated with 100 μc of ^{3}H-uridine per culture and actinomycin D as described in the legend to Fig. 1. The culture medium was then removed, the cells were washed with PBS without Ca or Mg, and 1 ml of 0.25% trypsin solution was added to each for 5 min of incubation at room temperature with occasional gentle agitation. The trypsin solution and floating cells were removed, and each dish was washed with 1 ml of PBS. The pooled trypsin and wash solutions were centrifuged at 4,000 rev/min for 5 min to remove whole cells; the supernatant fraction was layered over two sucrose layers (1 ml of 65% sucrose in D_2O and 5 ml of 15% sucrose) in a centrifuge tube and centrifuged for 2 hr at 25,000 rev/min and 4 C in the Spinco SW 25.3 rotor as previously described for virus purification (3). All of the particulate material which included whole virus and nucleocapsid at the interface between the sucrose layers was recovered and layered over a linear 15 to 65% sucrose density gradient and centrifuged for 12 hr at 25,000 rev/min and 4 C in a Spinco SW 25.3 rotor.*

component has the buoyant density of viral nucleocapsid, and electron microscopy of material from this region of the gradient reveals typical structures of viral nucleocapsid.

When ^{3}H-RNA was isolated from each component, it was found to sediment as 57*S* viral RNA. Thus, it appears that the action of trypsin resulted in release of both infectious virus and viral nucleocapsid from infected cells. After incubation of infected cells with H^3-uridine for 2 hr, significantly more radioactivity appeared in the RNA of the trypsin-released nucleocapsid than in trypsin-released whole virus.

Figure 8 shows a comparison of the effects of trypsin, neuraminidase, and EDTA on release of virus and nucleocapsid from infected cells. Identical cultures of infected cells after incubation with ^{3}H-uridine in the presence of actinomycin D, as described for Fig. 7, were treated with trypsin solution (Fig. 8A), *V. cholerae* neuraminidase at a concentration 10 times that required to inhibit agglutination of an equivalent number of chicken erythrocytes (Fig. 8B), or a versenate solution at a concentration which removes epithelial cells from culture dishes (Fig. 8C). The particulate material released by trypsin can again be seen to be fractionated by sucrose gradient centrifugation into two components corresponding to whole virus at a density of 1.23 g/ml and nucleocapsid at a density of 1.27 g/ml (Fig. 8A). Very small amounts of whole virus and almost no detectable nucleocapsid were released from cells by neuraminidase (Fig. 8B) or EDTA (Fig. 8C), indicating that the whole virus and nucleocapsid released with trypsin are not bound to cells by bonds disrupted by neuraminidase or EDTA.

Incubation of purified whole ^{3}H-virus with trypsin at room temperature and subsequent fractionation of the ^{3}H-labeled virus preparation by sucrose gradient sedimentation, as described for Fig. 7, demonstrated that no nucleocapsid was released from whole virus by trypsin alone. This indicates that nucleocapsid is released from cells rather than whole virus after incubation of infected cells with trypsin. Whether the nucleocapsid in these experiments comes from a few cells which are mechanically disrupted during the trypsin treatment and centrifugation, or whether trypsin more selectively releases nucleocapsid from sites near or at the surface of infected cells, is not completely clear. An experiment to test these possibilities suggests the latter. Cells were incubated with ^{3}H-uridine between 11 and 25 hr after infection in the absence of actinomycin D to permit cellular RNA, such as ribosomal RNA and virus-specific RNA, to become radioactive. The cells were then gently removed from the dishes with trypsin and collected by centrifugation. The total radioactive RNA in the cells was determined, a sample of the trypsin solution was used to determine total radioactivity released by trypsin, and a second sample was used to determine the radioactivity in the RNA in nucleocapsid and whole virus which were isolated from the

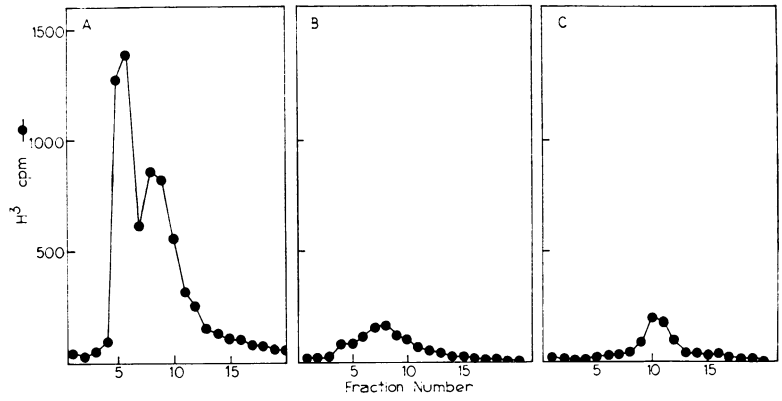

FIG. 8. *Particulate material released from cells by trypsin, neuraminidase, and EDTA. Six 100-mm cultures with 6 × 10^6 cells each were incubated with 100 μc of ^{3}H-uridine per culture and actinomycin D between 18 and 20 hr after infection, as described in the legend to Fig. 1. (A) The cells of two cultures were incubated with trypsin and washed as described in the legend to Fig. 7. (B) Fifty units of Vibrio cholerae neuraminidase in 1 ml was added to the second pair of cultures, which were then incubated at room temperature for 20 min. Neuraminidase and floating cells were removed, and 1 ml of PBS was used to wash each plate as in part A. (C) A 1-ml amount of 0.001 M sodium versenate in PBS without Ca^{2+} and Mg^{2+} was added to the third pair of cultures. The cultures were incubated for 20 min at room temperature, and the dishes were washed as for parts A and B. The supernatant fraction of each sample, which was centrifuged at 4,000 rev/min to remove whole cells, was then centrifuged in sucrose gradients as described in the legend to Fig. 7.*

trypsin solution in a sucrose density gradient, as in Fig. 7. Table 2 shows that about 1.5% of the total radioactive RNA of the cells was released into the trypsin solution, and, of the trypsin-released material, about 20% was RNA in nucleocapsid. We have also shown that the amount of radioactive nucleocapsid and virus released by trypsin is 15 to 20% of the amount of intracellular radioactive nucleocapsid (Fig. 9). Thus, a much greater proportion of total cell-associated radioactive nucleocapsid than total radioactive-cell RNA, most of which is ribosomal and transfer RNA, is released by trypsin. This indicates that trypsin preferentially or selectively frees nucleocapsid from infected cells and suggests that nucleocapsid may accumulate at or near the cell surface. In the case of SV5-infected monkey kidney cells, viral nucleocapsid has been shown to accumulate immediately under some areas of the cell surface (7).

Kinetics of virus assembly. We have described four structures containing 57S RNA which can be recovered from Sendai-infected cells or cell culture medium: intracellular nucleocapsid, nucleocapsid, virus released from cells by trypsin, and virus in the culture medium. An experiment was then done to determine the time course of ^{3}H-uridine incorporation into the RNA of each of these structures. Actinomycin D-treated cells were incubated with ^{3}H-uridine from 18 to 22 hr after infection, and the four particulate components named above were isolated at various times throughout this period. The cells were removed with trypsin as described for Fig. 7, and then cells were disrupted by a Dounce homogenizer without NP40. Virus in the medium and whole virus and nucleocapsid released by trypsin at each time were separated (Fig. 7), and the nucleocapsid was recovered from the disrupted cells (Fig. 1C). Figure 9 shows the results of this experiment, and it can be seen that ^{3}H-uridine first appears in cytoplasmic nucleocapsid; second, in nucleocapsid released by trypsin; third, in virus released by trypsin; and finally in virus in the culture medium. This order is compatible with the structures being formed in a stepwise sequence during virus assembly. In addition, during the experiment, intracellular nucleocapsid progressively accumulates, since the rate of its formation is significantly greater than its rate of conversion to virus.

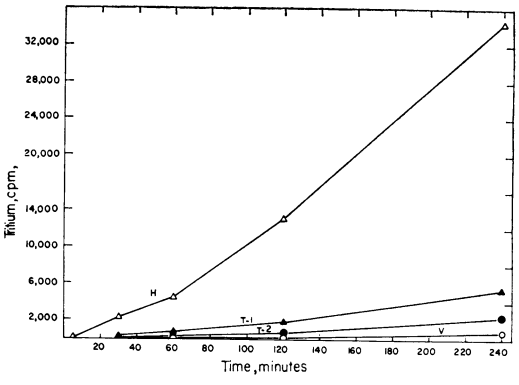

FIG. 9. *Incorporation of ^{3}H-uridine into four viral structures containing 57S RNA. Five milliliters of medium with 2 μg of actinomycin D per ml was added to each of five 100-mm cultures at 17.5 hr after infection with Sendai virus; 30 min later, 100 μc of ^{3}H-uridine was added to each culture, and incubation was continued at 37 C. The incubation of individual cultures was stopped 5, 30, 60, 120, and 240 min later by placing the dish in an ice bath and replacing the medium with ice-cold PBS. Cells were removed with 0.25% trypsin in the cold, collected by centrifugation, and disrupted by Dounce homogenization as described in the text. The culture medium, trypsin solution, and cytoplasmic extract for each incubation time were centrifuged in sucrose density gradients to isolate nucleocapsid and intact virus. The sum of the trichloroacetic acid-precipitable radioactivity in the nucleocapsid from the cytoplasmic extract or homogenate (H), nucleocapsid from the trypsin solution (T_1), intact virus from the trypsin solution (T_2), and intact virus from the culture medium (V) are plotted for each incubation time.*

TABLE 2. *Release of tritium-labeled RNA from infected cells by trypsin*[a]

Fraction	Total acid-precipitable ^{3}H-uridine (counts/min)
Total cell RNA	4,204,000
Total RNA released by trypsin	64,210
Nucleocapsid RNA released by trypsin	13,428

[a] Four 100-mm cultures were each incubated with 100 μc of ^{3}H-uridine in 5 ml of medium without actinomycin D from 11 to 25 hr after infection with Sendai virus. Cells were then removed with trypsin and collected by centrifugation as described in Fig. 7. The total RNA was extracted from cells and from one-half of the trypsin solution by the sodium dodecyl sulfate-phenol method; a sample was used for trichloroacetic acid precipitation and counting. The other half of the trypsin solution was centrifuged in sucrose density gradients for isolation of nucleocapsid and virus, as described in the legend to Fig. 7.

Serial electron micrographs of Sendai-infected cells (Fig. 10) document this accumulation. By 18 hr postinfection (Fig. 10A), large aggregates of viral nucleocapsid are evident in infected cell cytoplasm. By 4 days after infection (Fig. 10B), the size of nucleocapsid aggregates has enlarged

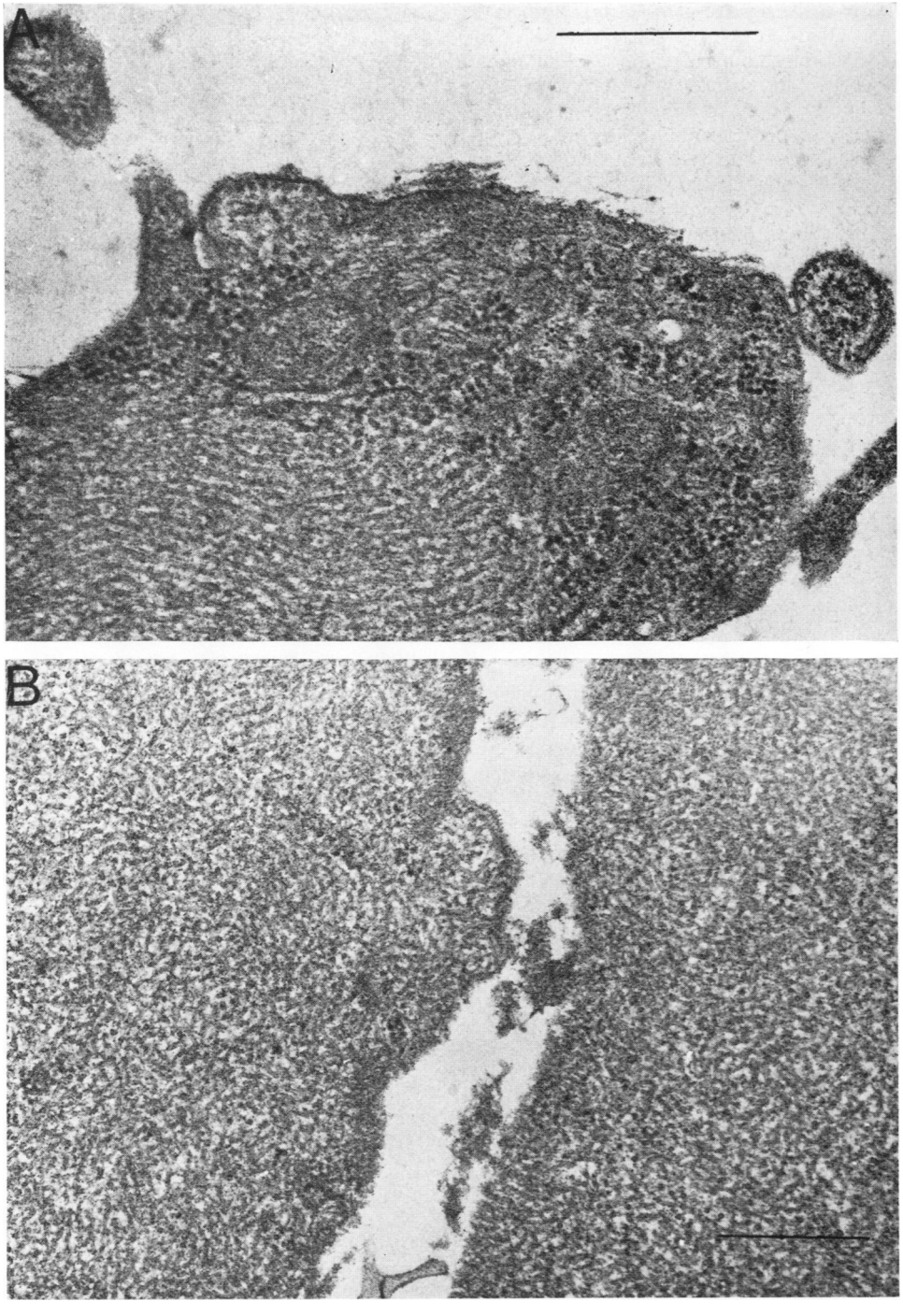

FIG. 10. *Electron microscopy of Sendai virus-infected chick embryo fibroblasts. Cells at 18 hr (A) and 4 days (B) were prepared for electron microscopy as described in the text. Magnification: A, × 54,000; B, 44,000.*

until much of the normal cytoplasm is displaced. In other subgroup 2 myxovirus-infected cells, such as SV5-infected BHK cells (7) and parainfluenza type 2-infected FL and HeLa cells (10), similar accumulation of viral nucleocapsid in infected cell cytoplasm has been shown.

These results indicate that, relative to the rate of viral RNA synthesis and its rapid conversion to nucleocapsid in Sendai virus-infected chick embryo fibroblasts, conversion of nucleocapsid to mature enveloped virus is a very slow or inefficient step in virus formation. The radioactivity data suggest that only approximately 5% of newly synthesized viral RNA leaves the cell in whole virus and the remainder progressively accumulates in the cell in the form of viral nucleocapsid. The low yield of infectious virus by cultures in which almost all cells are infected (2) and the serial electron micrographs showing large amounts of nucleocapsid within infected cell cytoplasm are in accord with this. With SV-5, another subgroup 2 myxovirus, there is evidence that this behavior is cell-specific (7, 8). The reason for the relative block in virus formation is not clear, but it could be due to a limiting rate of synthesis of an essential component of the virus, such as a viral envelope protein. Experiments are in progress to characterize the virion proteins and attempts will be made to learn whether any virus component is synthesized in limiting amounts.

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LITERATURE CITED

1. Baltimore, D., M. Girard, and J. E. Darnell. 1966. Aspects of the synthesis of poliovirus RNA and the formation of virus particles. Virology 29:179–189.
2. Blair, C. D., and W. S. Robinson. 1968. Replication of Sendai virus. I. Comparison of viral RNA and virus-specific RNA synthesis to Newcastle disease virus. Virology 35:537.
3. Bratt, M. A., and W. S. Robinson. 1967. Ribonucleic acid synthesis in cells infected with Newcastle disease virus. J. Mol. Biol. 23:1–21.
4. Bukrinskaya, A. G., S. M. Klimenko, J. A. Smirnov, and B. V. Guschin. 1968. Infective substructures of Sendai virus from infected Ehrlich ascites tumor cells. J. Virol. 2:752–758.
5. Compans, R. W., and P. W. Choppin. 1967a. Isolation and properties of the helical nucleocapsid of the parainfluenza virus SV5. Proc. Nat. Acad. Sci. U.S.A. 57:949–956.
6. Compans, R. W., and P. W. Choppin. 1967b. The length of the helical nucleocapsid of NDV. Virology 33:344–346.
7. Compans, R. W., K. V. Holmes, S. Dasel, and P. W. Choppin. 1966. An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV5. Virology 30:411–426.
8. Holmes, K. V., and P. W. Choppin. 1966. On the role of the response of the cell membrane in determining virus virulence; contrasting effect of the parainfluenza virus SV5 in two cell types. J. Exp. Med. 124:501–519.
9. Hosaka, Y., H. Kitano, and S. Ikeguchi. 1966. Studies on the pleomorphism of HVJ virions. Virology 29:205–221.
10. Howe, C., C. Morgan, C. deVaux St. Cyr, K. C. Hsu, and H. M. Rose. 1967. Morphogenesis of type 2 parainfluenza virus examined by light and electron microscopy. J. Virol. 1:215–237.
11. Hoyle, L. 1952. Structure of the influenza virus. The relation between biological activity and chemical structure of virus fractions. J. Hyg. 50:229–245.
12. Kingsbury, D. W., and R. W. Darlington. 1968. Isolation and properties of Newcastle disease virus nucleocapsid. J. Virol. 2:248–255.
13. Kuhn, N. O., and C. G. Harford. 1963. Electron microscopic examination of cytoplasmic inclusion bodies in cells infected with parainfluenza virus, type 2. Virology 21:527–530.
14. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:208–212.
15. Rott, R., I. M. Reda, and W. Schäfer. 1962. Isolation and characterization of hemagglutinating non-infectious particles produced during multiplication of NDV. Virology 16:207–209.
16. Weinberg, R. A., V. Loening, M. Willems, and S. Penman. 1967. Acrylamide gel electrophoresis of HeLa cell nucleolar RNA. Proc. Nat. Acad. Sci. U.S.A. 58:1088–1095.