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Cytoplasmic Compartmentalization of the Protein and Ribonucleic Acid Species of Vesicular Stomatitis Virus

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The cytoplasmic sites of synthesis in L cells of the protein and ribonucleic acid species of vesicular stomatitis virus were studied by polyacrylamide gel electrophoresis after fractionation of membrane and other cytoplasmic components by the Caliguiri-Tamm technique. The viral spike protein (glycoprotein G) was found primarily associated with a smooth membrane fraction which is rich in plasma membrane; the G protein was also present in fractions containing rough endoplasmic reticulum. The nonglycosylated envelope protein S (also called M) was found in the smooth membrane fractions but was more abundant in endoplasmic reticulum-enriched fractions. Longer labeling resulted in detection of nucleoprotein N, as well as other minor nucleocapsid proteins L and NS_1, in the cellular membrane fractions. The N protein appeared to be made in membrane-free cytoplasm along with progeny ribonucleic acid and later became associated with membrane containing G and S viral proteins.

We have previously reported (18) that the two envelope proteins of vesicular stomatitis (VS) virus are synthesized in L cells in association with a sedimentable cellular component. Both the glycoprotein (G) and the membrane protein (S, or M in the new nomenclature) could be solubilized by the membrane-dissolving sterol glycoside, digitonin, whereas the third major structural protein (N) could not. [This manuscript was prepared before workers in this field agreed at the 1971 Gordon Research Conference on Animal Cells and Viruses to designate the small, nonglycosylated membrane protein of VS virus as the M protein. It was also decided that the other structural proteins be designated L, G, and N (18).] Based on these data, we advanced the hypothesis that the glycoprotein and the nonglycosylated membrane protein are synthesized at and inserted into plasma membrane of the infected cell, but the nucleoprotein is synthesized in the cytoplasm in association with virion ribonucleic acid (RNA). Cohen et al. (6) recently presented convincing evidence that the VS viral G and S envelope proteins (proteins II and V in their terminology) are synthesized in association with the plasma membrane, but the nucleoprotein (core protein III) attaches later to the plasma membrane.

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The experiments reported here confirm the initial observations of our two laboratories and provide additional evidence that the virion components of VS virus are synthesized at different cellular sites before assembly at the plasma membrane.

MATERIALS AND METHODS

Virus, cells, and media. These materials were similar to those used in previous experiments (13, 18). The Indiana serotype of VS virus was used throughout. Infectivity titers per milliliter of stock inocula did not vary significantly from 10^9 to 2×10^9 plaque-forming units (PFU). For certain experiments, infectious B virions were purified by rate zonal sedimentation in sucrose gradients (10). Monolayer cultures of L cells were grown to confluency in plastic petri plates (100 mm in diameter) with medium 199 and 10% calf serum. Primary chick embryo (CE) cells were prepared from minced, trypsinized 9-day-old chicken embryos and were propogated in 100-mm plates in 0.5% lactalbumin hydrolysate supplemented with 5% fetal calf serum. Media used for experimental infection were Eagle basal medium (BME) with or without leucine and tyrosine, special BME without glucose but with twice the content of amino acids, or Eagle minimal essential medium (MEM), all purchased from Grand Island Biological Co., Grand Island, N.Y.

Radioisotopes. ^{3}H-leucine (58 Ci/mmole), ^{3}H-tyrosine (50 Ci/mmole), ^{3}H-uridine (28 Ci/mmole), ^{3}H-glucosamine (3.6 Ci/mmole), and reconstituted

uniformly labeled ^{14}C-amino acids (54 mCi/matom) were obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y.

Infection and labeling of L cells. In a typical experiment, a set of 15 monolayer cultures of L cells ($\sim 10^7$ cells/culture) were infected with VS virus at a multiplicity of 20 to 50 PFU/cell. Virus was allowed to adsorb for 30 min at 37 C, and the cells were incubated in 5 ml of medium specially selected for each labeling condition. At 2.5 hr after adsorption, actinomycin D was added to the medium in each culture to give a final concentration of 4 μg/ml. Each set of 15 cultures was labeled by adding appropriate ^{3}H or ^{14}C precursors at 3.5 hr postinfection as follows: ^{3}H-leucine plus ^{3}H-tyrosine (5 to 10 μCi/ml each) in special BME devoid of unlabeled leucine, tyrosine, and serum, or ^{3}H-uridine (10 μCi/ml) in MEM with 5% fetal calf serum. At 5 hr postinfection (90-min labeling), infected cell cultures were harvested, fractionated, and analyzed.

Cell fractionation. The procedure used was basically that of Caliguiri and Tamm (5). Labeled, infected (or uninfected) monolayer cultures were washed twice with cold phosphate-buffered saline (PBS); the cells in each culture were scraped with a rubber policeman into 5 ml of Earle's balanced salt solution (BSS) and pooled for each set of cultures. The suspended cells were then centrifuged, washed with BSS, and repelleted at $2,000 \times g$ for 5 min. The washed pellet of $\sim 15 \times 10^7$ cells was then suspended in 4 ml of reticulocyte standard buffer (RSB) consisting of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.01 M NaCl, 0.0015 M $MgCl_2$ (pH 7.4). After allowing the cells to swell for 20 min with intermittent dispersal with a Vortex mixer, they were disrupted by 15 or 20 strokes of a tight-fitting pestle in a 7-ml Dounce homogenizer. The nuclei and large subcellular fragments were removed by centrifugation at $850 \times g$ for 10 min. Monitoring samples by phase microscopy revealed that this procedure removed all visible nuclei and intact cells and left many small fragments of membrane in the cytoplasm. Some intact sheets of plasma membrane were pelleted with the nuclear fraction but much of the disrupted membrane remained in the supernatant fluid.

The cytoplasmic suspension containing membrane fragments was then fractionated by centrifugation in a discontinuous sucrose gradient (5) prepared by successive layering of the following sucrose solutions (w/w) in RSB, pH 7.4: 3 ml of 60%, 7 ml of 45%, 7 ml of 40%, 10 ml of the cytoplasmic suspension made 30% with respect to sucrose, 7 ml of 25%, and 3 ml of RSB. The gradients were centrifuged at $96,000 \times g$ for 18 to 19 hr at 4 C. Light-scattering bands, usually four in number, were collected from the side of the tube with a needle and syringe. The final fractionation step was to concentrate each fraction by centrifugation at $80,000 \times g$ for 90 min in a type 30 rotor. Each pellet was suspended in 0.5 ml of RSB, except for those to be examined in the electron microscope.

Electron microscopy. The sucrose gradient fractions of infected and uninfected L cell cytoplasm were prepared for electron microscopy essentially as described previously (16, 17). Unfixed pellets were suspended in phosphotungstic acid (PTA) and examined for the presence of negatively stained viral nucleocapsids and virions. Other specimens were prepared for sectioning by pelleting in a microfuge tube filled with buffered glutaraldehyde, fixed in OsO_4, stained with uranyl acetate, embedded in Epon-812, sectioned, post-stained with 1% uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 1A electron microscope at an initial magnification of $\times 40,000$.

Enzyme markers for cytoplasmic components. The cytoplasmic components fractionated in sucrose gradients were assayed for the presence of the following enzyme activities. The activity of the enzyme reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome c reductase, assayed by the method of Sottocasa et al. (15), was used as indication of the presence of endoplasmic reticulum. The method described by MacGregor and Schnaitman (11) to measure the activity of succinic dehydrogenase was used to mark the presence of mitochondrial membrane. Na^+, K^+-stimulated adenosine triphosphatase was assayed by the method of Avruch and Wallach (2). Inhibition of the adenosine triphosphatase by 0.1 mM ouabain provided a basis for identifying those cytoplasmic components rich in plasma membrane (2, 4).

Extractions and analysis of proteins. Samples of 100 μliters of ^{3}H-protein in cytoplasmic fractions were made 1% with respect to sodium dodecyl sulfate (SDS) and 2-mercaptoethanol and were mixed with 20 μliters of marker ^{14}C-proteins extracted from purified VS virions. This mixture was heated in a boiling water bath for 2 min; 5 μliters of 1% bromphenol blue was then added as marker as well as sucrose to a final concentration of 10%. As previously described (13, 18), migration of labeled proteins was determined by electrophoresis in 7.5% polyacrylamide gels at 5 ma/gel for 6.5 hr. Gels sliced into lengths of 1.25 mm were dissolved in 0.5 ml of Nuclear-Chicago solubilizer at 50 C for 2 hr and diluted in toluene-based fluors; disintegrations of ^{3}H-protein extracted from infected cell fractions and marker ^{14}C-proteins were counted by scintillation spectrometry assisted by a double-labeling computer program.

Extraction and analysis of RNA. The RNA extraction procedure was similar to that described by Duesberg and Blair (7). Cytoplasmic extracts in RSB were made 1% with respect to SDS, and to this was added an equal volume of 90% redistilled phenol in RSB. After shaking for 5 min, the solution was chilled in an ice bath and centrifuged at $2,000 \times g$ at 4 C for 10 min. The aqueous phase was drawn off and the RNA was re-extracted with one-third volume of 90% phenol. The RNA was precipitated from the aqueous phase by adding two volumes of cold ethanol. After 12 hr at -20 C, the RNA precipitate was pelleted at $10,000 \times g$ for 10 min, washed once with ice-cold 70% ethanol, and suspended in NTE buffer [0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.01 M Tris, pH 7.4] containing 0.5% SDS and 15% sucrose.

The RNA was analyzed by electrophoresis in

polyacrylamide gels prepared in electrophoresis buffer (0.02 M sodium acetate, 2 mM EDTA and 0.04 M Tris, *p*H 7.8) containing 2.3% acrylamide and 0.12% *N,N'*-bis-methylene acrylamide. The gels were polymerized with ammonium persulfate in Plexiglas tubing (0.7 by 12 cm) with dialysis tubing stretched over the bottom to prevent the gel from slipping out. Sodium acetate electrophoresis buffer was used in both the upper and lower reservoirs, and the gels were pre-electrophoresed for 1 hr. Samples of ^{3}H-RNA extracted from virus-infected cells were mixed with marker ribosomal ^{14}C-RNA in 0.5% SDS and 15% sucrose in NTE buffer. RNA samples were applied to the gels in amounts of 50 to 200 μliters which contained 5 to 50 μg of RNA. Electrophoresis was for 170 min at 5 ma/gel and 4 C. After electrophoresis, the gels were wrapped in aluminum foil, frozen at −70 C, extruded from the plexiglas tube, and sectioned in the frozen state into 1.5-mm slices with a spaced series of razor blades. The gel slices were depolymerized with Nuclear-Chicago solubilizer, and their radioactivities were counted as described above for proteins.

RESULTS

Electron microscopic identification of the fractionated cell components. Uninfected and infected monolayers containing a total of ~15 × 10^7 L cells were disrupted by Dounce homogenization, and the cytoplasm plus membrane portion was fractionated by centrifugation on a 0 to 60% discontinuous sucrose gradient as described above. The cytoplasm of infected cells consistently separated into at least four light-scattering bands when centrifuged for 18 hr in this gradient. The top band 1 appeared in the region of the interface of 25 and 30% sucrose, band 2 at the interface of 30 to 40% sucrose, band 3 at the 40 to 45% sucrose interface, and a very granular band 4 at the 45 to 60% sucrose interface. Band 3 was usually absent in extracts of uninfected cells. Occasionally, top band 1 was partially dissociated into two adjacent bands as was band 2 on occasion. However, the same components were found in the split bands and they are, therefore, each considered as a single band. Pelleted material from each band was fixed, stained, section, and examined by electron microscopy or examined unfixed by negative staining with PTA.

Representative electron micrographs of sectioned preparations of fractionated cytoplasmic components of VS virus-infected L cells are shown in Fig. 1 to 3. The least dense band 1 was composed predominantly of smooth membrane (Fig. 1). Band 2, shown at a higher magnification to demonstrate the presence of ribosomes, had the appearance of rough endoplasmic reticulum (Fig. 2) but did contain some smooth membrane fragments. The densest component, band 4, was composed of rough endoplasmic reticulum (Fig. 3) plus some free ribosomes. Sections of band 3 revealed only amorphous material, but PTA negative staining of unfixed material revealed a predominance of nucleocapsid coils (17). Not shown in the figures were occasional mitochondria in bands 3 and 4 as well as occasional mature virions in sections of bands 1 and 2 of infected cells. Sucrose gradient fractions of uninfected L cells were identical in appearance except for the absence of virions and viral nucleocapsids.

From these observations, which are similar to those of Caliguiri and Tamm (5) with HeLa cells, we conclude that the predominant cytoplasmic components of fractionated L cells infected with VS virus are as follows: band 1, smooth membrane; band 2, primarily rough endoplasmic reticulum; band 3, viral nucleocapsids; and band 4, rough endoplasmic reticulum and free ribosomes.

Distribution of enzyme markers in fractionated components of L cells. To categorize further the membrane components identified by electron microscopy of fractionated L cell cytoplasm, the activity of various enzymes was measured. The activity of Na^+, K^+-stimulated adenosine triphosphatase which was sensitive to ouabain provided a basis for locating enrichment with plasma membrane (2, 4). In addition, the presence of endoplasmic reticulum was monitored by assays of NADPH-cytochrome *c* reductase (15) and that of mitochondrial membrane by succinic dehydrogenase (11).

Two sets of 15 cultures of L cells were each labeled for 12 hr with a mixture of ^{14}C-amino acids (3.5 μCi/ml). One set of cultures was then infected with VS virus at a multiplicity of ~50, and the other set was not infected. All cultures were then labeled for 3 hr with 10 μCi each of ^{3}H-leucine and ^{3}H-tyrosine per ml. The cells from each set of cultures were then washed and disrupted by Dounce homogenization, and the cytoplasmic suspension was fractionated by centrifugation in a 0 to 60% discontinuous sucrose gradient as described above. Twenty 2-ml fractions were collected from each gradient and analyzed for radioactivity (protein) and for the presence of various enzyme activities.

Figure 4 shows the distribution of protein across the sucrose gradient and the relative activity of each enzyme. Corresponding analysis of the fractionated cytoplasmic and membranous components of VS virus-infected L cells showed almost identical patterns of enzyme distribution as well as superimposable distribution of pre-infection ^{14}C-protein and postinfection ^{3}H-protein. As noted, bands 2 and 4 appear to be

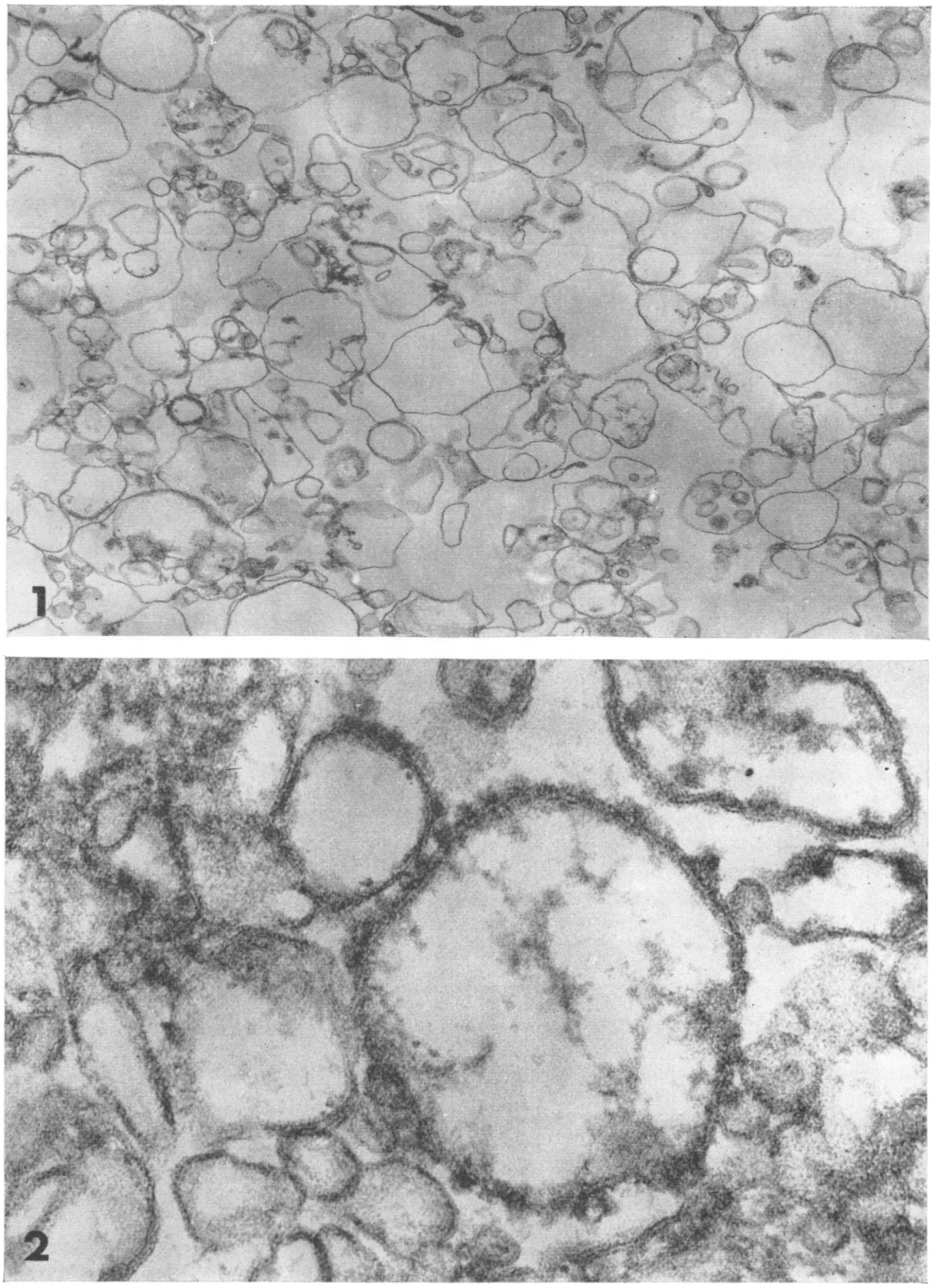

FIG. 1–2. *Sections of fractionated components of infected L cells separated on a discontinuous gradient of 0 to 60% sucrose. Fig. 1, band 1 showing smooth membrane. × 25,000. Fig. 2, band 2 showing membrane with ribosomes. × 140,000.*

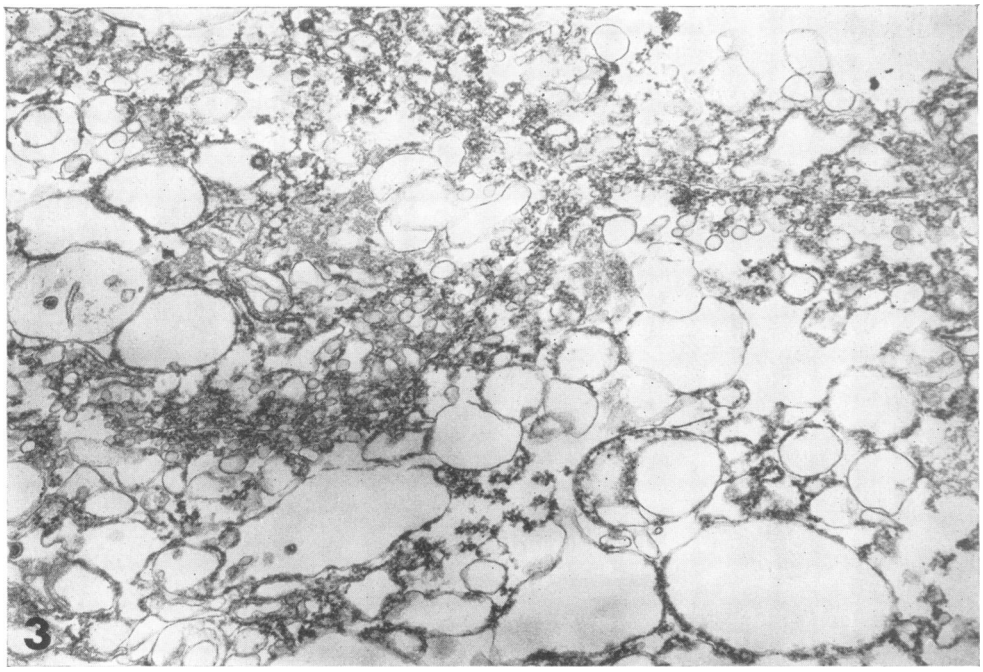

FIG. 3. *Section of fractionated components of infected L cells separated on a discontinuous gradient of 0 to 60% sucrose. Band 4, ribosomes and rough endoplasmic reticulum.* × *25,000.*

rich in endoplasmic reticulum as determined by the presence of NADPH-cytochrome *c* reductase (Fig. 4B). Succinic dehydrogenase activity is greatest in band 4 (Fig. 4C). The evidence that band 1 contains much of the plasma membrane is indicated by the relatively high concentration of ouabain-sensitive adenosine triphosphatase (Fig. 4D) compared with the lower concentrations of other enzymes. Total adenosine triphosphatase activity (Fig. 4E) was present in all three membrane fractions.

Comparative incorporation of labeled precursors of protein, RNA, and carbohydrate into fractionated components of L cells infected with VS virus. Evidence was sought of the biosynthetic capabilities of the fractionated membrane-cytoplasm components of infected L cells. To this end, three separate sets of L cell cultures were infected with VS virus at a multiplicity of ~50, exposed to actinomycin D (4 μg/ml), and labeled from 3.5 to 5 hr postinfection with ^{3}H-uridine (10 μCi/ml), ^{3}H-leucine plus ^{3}H-tyrosine (each 10 μCi/ml), or ^{3}H-glucosamine (4 μCi/ml). Cells harvested from each set of cultures were disrupted by Dounce homogenization and fractionated by isopyknic centrifugation in a 0 to 60% discontinuous sucrose gradient. Four light-scattering bands were collected from each gradient, their densities were measured by weighing in 50-μliter pipettes, and total radioactivity of acid-precipitable material was measured by scintillation spectrometry after precipitation and washing on nitrocellulose filters with 10% trichloroacetic acid. Appropriate controls revealed that under these conditions of labeling in the presence of actinomycin D, at least 99% of ^{3}H-uridine was incorporated into viral RNA and >90% of labeled amino acids was incorporated into viral proteins. Infection with VS virus at high multiplicity rapidly switches off most of cellular RNA and protein synthesis (20).

Table 1 shows the proportion of acid-precipitable radioactivity of the three precursors incorporated into the cytoplasmic fractions in each of the four bands in gradients of sucrose ranging in density from 1.12 to 1.22 g/ml. As expected, band 3, which by electron microscopy is composed largely of viral nucleocapsids (16, 17), contained almost 50% of the viral RNA, about one-third of the protein, but only 10% of the acid-precipitable carbohydrate. In contrast, band 1, the smooth membrane fraction, incorporated more than 50% of the total ^{3}H-glucosamine, 20% of the ^{3}H-amino acids, and less than 10% of the ^{3}H-uridine. In all likelihood, most of the ^{3}H-glucosamine incorporated into plasma membrane was present in host cell glycoprotein

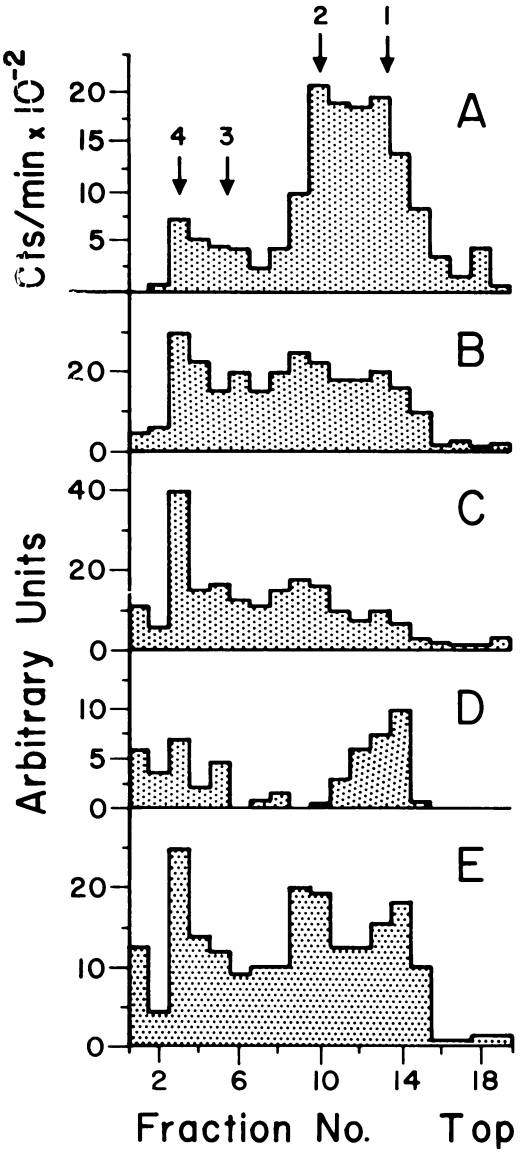

FIG. 4. *Distribution of protein and various enzyme markers of uninfected L cells fractionated by centrifugation in a 0 to 60% sucrose gradient. (A) Total protein, as indicated by incorporation of ^{14}C-amino acids. (B) NADPH-cytochrome c reductase activity as an indication of the presence of endoplasmic reticulum. (C) Succinic dehydrogenase levels as an indication of mitochondrial membrane. (D) Distribution of oubain-sensitive Na^+,K^+-adenosine triphosphatase. (E) Total Na^+,K^+-adenosine triphosphatase. Cells infected with VS virus (data not shown) revealed the same distribution of protein and the various enzyme markers as the uninfected control cells.*

TABLE 1. *Distribution of trichloroacetic acid-insoluble radioactivity in the four light-scattering bands obtained by centrifuging a cytoplasmic extract of vesicular stomatitis (VS) virus-infected L cells in a 0 to 60% discontinuous sucrose gradient*[a]

Precursor	Per cent radioactivity				Total counts per min
	Band 1[b]	Band 2	Band 3	Band 4	
^{3}H-uridine	9.1	17.1	47.0	26.8	144,480
^{3}H-leucine + tyrosine	20.0	24.9	34.2	20.9	182,850
^{3}H-glucosamine	55.5	16.8	10.6	17.1	15,150

[a] Monolayers of L cells were infected with VS virus at an input multiplicity of 20 plaque-forming units per cell. For labeling the protein or glycoprotein, actinomycin D was added 30 min prior to infection; when ^{3}H-uridine was used, the actinomycin was added at 2.5 hr postinfection. The three groups (13×10^7 cells/group) were labeled from 3.5 to 5 hr postinfection with either ^{3}H-uridine (10 μCi/ml), ^{3}H-leucine plus ^{3}H-tyrosine (10 μCi/ml of each), or ^{3}H-glucosamine (4 μCi/ml). At 5 hr postinfection, cells were harvested and disrupted in a Dounce homogenizer, and the cytoplasmic extract was centrifuged on a 0 to 60% (w/w) discontinuous sucrose gradient for 18 hr at 96,000 × *g* at 4 C. Four light-scattering bands were removed by needle from the side of the tube, and the density of each band was determined by weighing 50-μliter quantities. Each band was then diluted with reticulocyte standard buffer [RSB; 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.01 M NaCl, 0.0015 M $MgCl_2$ (*p*H 7.4)] and pelleted at 80,000 × *g* for 90 min in a type 30 rotor. The pellets were resuspended in 2.5 ml of RSB, and 0.2 ml was precipitated on nitrocellulose filters by 10% trichloroacetic acid after addition of 200 μg of bovine serum albumin carrier. Filters were washed twice with cold 5% trichloroacetic acid and placed in counting vials. These were placed in a water bath at 100 C after the addition of 0.5 ml of 4% trichloroacetic acid to each vial. After 30 min, the vials were removed, cooled, and counted as described in Materials and Methods.

[b] Density of sucrose gradient fractions (grams per milliliter): band 1, 1.12; band 2, 1.17; band 3, 1.20; band 4, 1.22.

and glycolipids; infection with VS virus does not switch off glycosylation of cellular glycoproteins and glycolipids (R. R. Wagner, *unpublished data*). Bands 2 and 4 of the sucrose gradients did not reveal any significant differences in distribution of RNA, protein, and carbohydrate. Similar total amounts of ^{3}H-glucosamine and ^{3}H-amino acids were incorporated into uninfected cells exposed to actinomycin D, but ^{3}H-uridine incorporation into the four fractions of

uninfected cells was about 1% of that in the same number of cells infected with VS virus.

These data suggest that VS viral proteins are synthesized in all the cytoplasmic components, but that much of the ^{3}H-amino acids and ^{3}H-uridine was used for synthesis of viral nucleocapsids unassociated with cell membranes. In contrast, most of the ^{3}H-glucosamine was incorporated into smooth membrane constituents, although not necessarily of viral origin.

Cellular distribution of specific VS viral proteins. Cohen et al. (6) have demonstrated that the G and S envelope proteins of VS virus are preferentially associated with plasma membrane of infected HeLa cells subjected to a short pulse of labeled amino acids. In their studies, the nucleoprotein (N) was detectable in the plasma membrane fraction only after a 30-min chase. We have essentially confirmed these observations despite considerable difficulty in doing these experiments with L cell monolayers. In our system, incorporation of labeled amino acids is less efficient, and large cellular pools of amino acids preclude reliable chase. Therefore, we have concentrated our efforts in studying the proportional distribution of each viral protein present in the Caliguiri-Tamm compartments of infected L cells after more prolonged labeling. These cell fractionation studies provide some basis for examining the distribution of viral protein species associated and unassociated with smooth and rough membranes.

Figure 5 shows the distribution of VS viral proteins among each of the four bands of fractionated L cells separated on a 0 to 60% discontinuous sucrose gradient. The cells were labeled with ^{3}H-amino acids for 90 min prior to harvesting at 5 hr after infection at a multiplicity of 50. Band 1 (smooth membrane) contained primarily G protein and smaller amounts of proteins N, S, and L. Band 2 was also rich in G protein but contained larger amounts of proteins N, S, and L (as well as NS_1) than did band 1. Band 3 (nucleocapsid cores) contained only proteins N, L, and NS_1. This finding is identical to that of L. Prevec (*personal communication*) who found the same three viral proteins in essentially the same proportions in VS viral nucleocapsids. Fraction 4 contained all the proteins in roughly the same proportions as were found in whole infected cells, S protein being the smallest component.

The results of a similar experiment in which infected L cells were labeled with ^{14}C-amino acids for a shorter period (30 min) are shown in Fig. 6. Under these conditions, virtually the only viral protein in the smooth membrane of band 1 was the glycoprotein. The glycoprotein was also the predominant viral protein in the rougher membrane of band 2 which also contained a considerable amount of protein S and smaller amounts of proteins N, NS_1, and L.

These experiments indicate that the glycoprotein is the primary VS viral protein associated with the cytoplasmic membrane-enriched smooth fraction of infected L cells. The other envelope protein (S) is also associated with this smooth membrane fraction, but appears to be present earlier and in larger amounts in a more dense membrane fraction which is enriched in endoplasmic reticulum. The nucleoprotein appears first and in largest amount in the nucleocapsid and only later becomes associated in appreciable amount with the cellular membrane components.

Glucosamine labeling of infected CE cell components. Another method for analyzing the cellular distribution of the VS viral glycoprotein is to measure incorporation of specific sugar precursors. ^{3}H- or ^{14}C-glucosamine is incorporated into the G protein but not the L, N, and S proteins of virions released from infected L cells (13, 18). However, we have had considerable difficulty in sugar labeling of viral glycoprotein in infected L cells because of poor uptake in these cells as well as considerable incorporation of label into normal cellular glycoproteins. VS virus inhibits cellular protein synthesis (20) but does not switch off glycosylation of preexisting cellular glycoproteins (13; R. R. Wagner, *unpublished data*). Far better results were obtained by the use of CE cells rather than L cells.

Fifteen monolayer cultures of $\sim 2 \times 10^7$ CE cells each were infected with VS virus at a multiplicity of $\sim$50 PFU/cell. At 5.5 hr postinfection, the cells were labeled for 30 min with ^{3}H-glucosamine (4 μCi/ml) in special BME without glucose but containing twice the usual concentration of amino acids (13). The cells were then washed, scraped into RSB, disrupted by Dounce homogenization; the homogenate freed of nuclei by centrifugation at 800 $\times$ g was fractionated on a 0 to 60% discontinuous gradient as described above. Four light-scattering bands were readily visible in the gradient at positions identical to those of fractionated L cells (see Fig. 5). Proteins extracted from each fraction with SDS were analyzed on polyacrylamide gels.

Figure 7 shows the distribution of ^{3}H-glucosamine label in the electropherograms of each banded component of VS viral-infected CE cells labeled for 30 min. There is a considerable amount of ^{3}H label at the top and bottom of each gel, presumably representing cellular glycoproteins and glycolipids. However, a major

peak of ^3H radioactivity extracted from bands 1 and 2 migrates to the same position as viral marker ^{14}C-glycoprotein. A lesser amount of viral ^3H-glucosamine was present in the dense rough ER of band 4. Band 3, the viral nucleocapsid fraction, was virtually free of viral glycoprotein. The specificity of ^3H-glucosamine incorporation was evident from the negligible amount of ^3H radioactivity in the gel regions marked by proteins N and S. CE cells labeled with ^3H-amino acids showed protein distribution in the fractionated components identical to that of L cells as shown in Fig. 5 and 6.

These experiments confirm the presence of VS viral glycoprotein in membrane components of infected cells, particularly in the smooth mem-

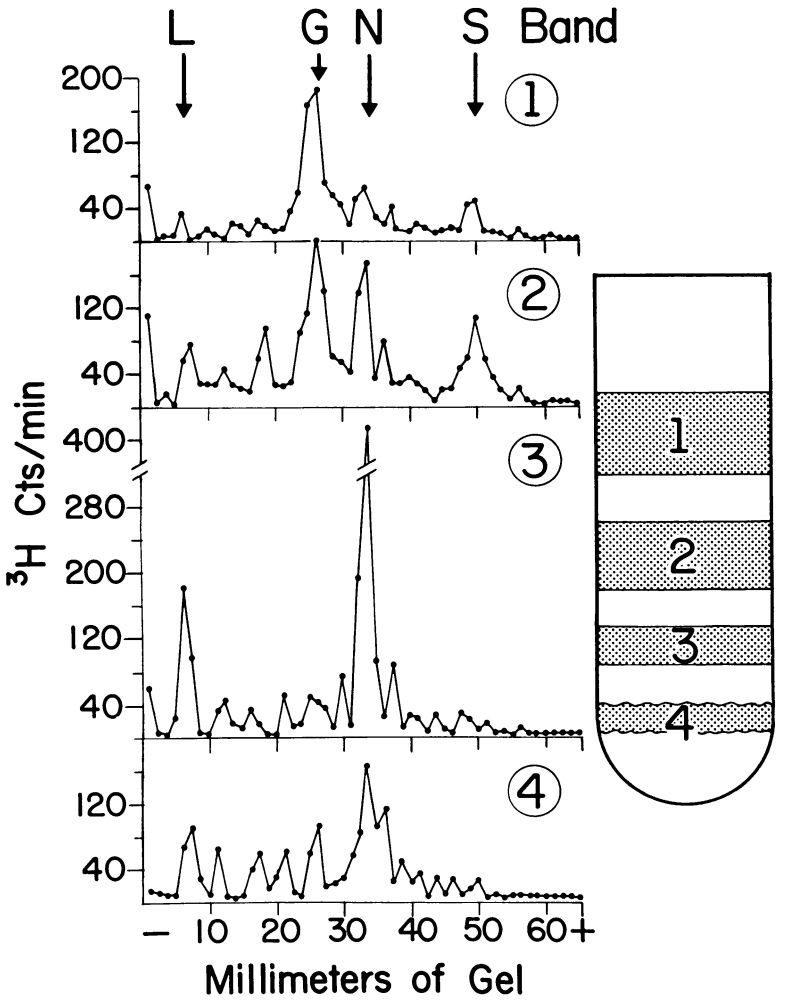

FIG. 5. *Electropherograms demonstrating differential association of VS viral proteins with the membranes and other cytoplasmic components of infected L cells fractionated by sucrose gradient centrifugation. Fifteen monolayer cultures each containing about 8 × 10^6 L cells infected with VS virus at a multiplicity of ~50 were incubated for 3.5 hr with special BME without serum, leucine, or tyrosine but containing actinomycin D (4 μg/ml) and then labeled with ^3H-leucine and ^3H-tyrosine (5 μCi/ml each) for 90 min. At 5 hr postinfection, the cells were washed, scraped into BSS, washed again, suspended in RSB, allowed to swell for 30 min, and disrupted in a Dounce homogenizer. The suspended membrane fragments and cytoplasmic organelles after removal of nuclei at 800 × g were centrifuged for 18 hr at 96,000 × g in a discontinuous 0 to 60% sucrose gradient. Light-scattering bands at the positions shown were collected by needle puncture of the side of the tube; the particulate material in each band was pelleted at 80,000 × g for 90 min. The proteins in each pelleted band were extracted by boiling in 1% SDS and electrophoresed in 7.5% polyacrylamide gels along with ^{14}C-protein markers extracted from purified VS virions. Arrows indicate the location in the gels of co-electrophoresed L, G, N, and S marker ^{14}C-proteins.*

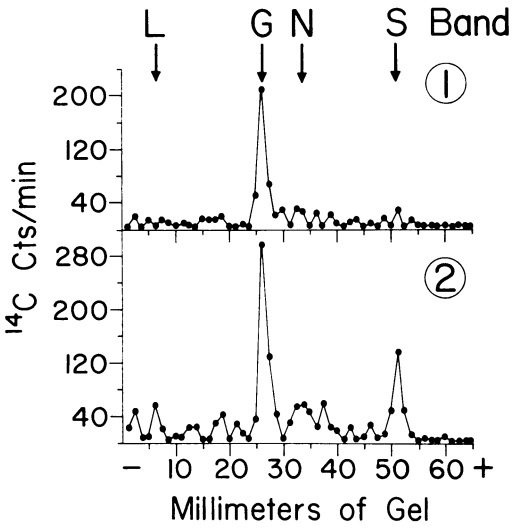

FIG. 6. *Electropherograms of infected cell membranes showing preferential association of viral envelope proteins G and S with smooth membrane (band 1) and endoplasmic reticulum (band 2). The experimental protocol was essentially the same as that described in the legend to Fig. 5 except that the medium for each culture consisted of 5 ml of MEM with 4% fetal calf serum and the cultures were labeled with ^{14}C-amino acids (1.3 μCi/ml) at 4 hr after infection and 1 hr after adding actinomycin D (4 μg/ml). The viral proteins in this experiment were extracted with acetic acid, urea, and SDS and were analyzed by electrophoresis on 7.5% polyacrylamide gels containing 0.1% SDS and 0.5 M urea. Arrows designate the peak regions of co-electrophoresed marker ^{3}H-proteins L, G, N, and S extracted from purified VS virions.*

brane fraction rich in plasma membrane and the absence of glycoprotein associated with nucleocapsids.

Cellular distribution of VS viral RNA. As many as nine species of VS viral RNA can be detected in actinomycin-treated cells infected under various conditions (9). Three of these viral RNA species appear to be incorporated into VS virions of different lengths and have been assigned approximate sedimentation coefficients as follows: 40 to 42S RNA in infectious B virions, 28 to 32S RNA in defective long T particles, and 20 to 23S RNA in defective short T particles (9, 12, 14, and Kiley and Wagner, *unpublished data*). In addition, a heterogeneous group of messenger RNA (mRNA) species complementary to virion RNA has been found in infected cells; the complementary mRNA appears to fall into at least two size classes, assigned approximate values of 28S and 13S (9, 12, 14). It was of interest to determine which species of viral RNA are associated with the membrane and other cytoplasmic components of infected L cells fractionated on discontinuous sucrose gradients.

These experiments on viral RNA distribution were performed in a manner similar to those in which viral proteins were studied except that short pulse labeling with ^{3}H-uridine gave more reliable results. L cells were infected at a multi-

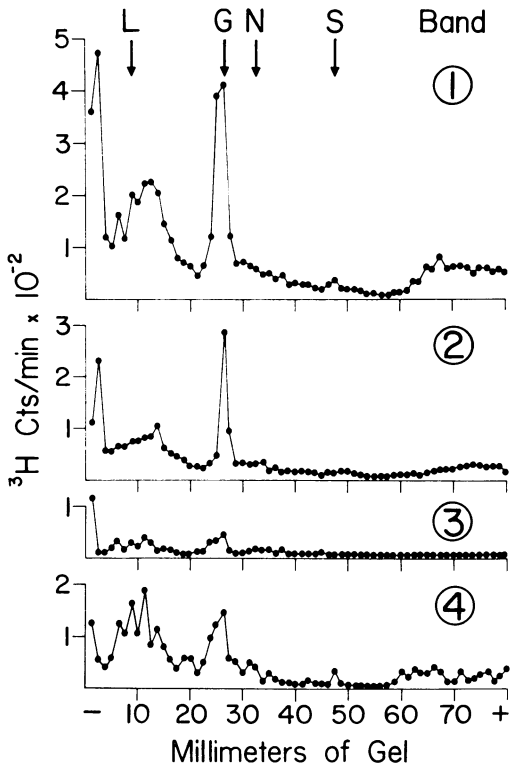

FIG. 7. *Electropherograms of VS viral-infected CE cells labeled with ^{3}H-glucosamine and fractionated by sucrose gradient centrifugation into smooth membrane (band 1), rough membrane (band 2), nucleocapsid fraction (band 3), and rough membranes plus ribosomes (band 4). Fifteen monolayer cultures of primary CE cells (~2 × 10^{7} cells/culture) were infected with VS virus at a multiplicity of 50. After 5.5 hr, the cells were labeled by adding ^{3}H-glucosamine (4 μCi/ml) in BME devoid of glucose but with twice the usual amount of amino acids. The cells were not exposed to actinomycin. As described in Materials and Methods and the legend for Fig. 5, the cells were suspended in RSB and disrupted by 20 strokes of a tight Dounce homogenizer; the cytoplasm plus membranes were fractionated by centrifugation for 18 hr at 96,000 × g. The proteins in each light-scattering band were solubilized in SDS and analyzed by acrylamide gel electrophoresis along with marker ^{14}C-proteins L, G, N, and S (arrows) extracted from purified VS virions.*

plicity of 20 with infectious B virions of VS virus purified by sedimentation in sucrose gradients to minimize contamination with defective T particles (9, 10). In our system, the predominant species of viral RNA found in infected L cells is 28S virion RNA (i.e., not complementary by hybridization to 42S virion RNA), most of which is present in ribonuclease-resistant nucleocapsid (Kiley and Wagner, *manuscript to be published*). The most reproducible results were obtained by pulse labeling with ^{3}H-uridine for 10 min at 2.5 hr postinfection or by prolonged labeling. True pulse-chase experiments were not feasible because of a large cytoplasmic pool of ^{3}H-uridine. Cells disrupted by Dounce homogenization were freed of nuclei and fractionated in discontinuous gradients of 0 to 60% sucrose, as described above; the RNA species extracted from the banded components with SDS and phenol were electrophoresed in 2.3% polyacrylamide gels along with ribosomal ^{14}C-RNA markers.

Figure 8 shows the electrophoretic profiles of viral ^{3}H-RNA extracted from each of the four bands of fractionated L cells pulsed with ^{3}H-uridine for 10 min at 2.5 hr after infection. Bands 1 and 2 (smooth and rough membrane) showed a heterogeneous group of RNA species. Band 3 (nucleocapsid) contained a predominant species of 28S RNA, as did band 4 (rough ER) to some extent. Moreover, all bands (but band 2 and 4 in particular) contained many size classes of RNA which could have been nascent mRNA species attached to polysomes or, of course, degraded viral RNA.

Figure 9 compares the electropherograms of the RNA species extracted from the four banded components of L cells infected with VS virus and labeled with ^{3}H-uridine between 3.5 and 5 hr postinfection. Under this condition of labeling, far more viral ^{3}H-RNA was present, and the predominant species in all four bands was 28S RNA. Also present in each of the four cell fractions was a smaller but quite definite peak of 42S RNA, which probably represents the species present in infectious B virions. Most of the remaining radioactivity in the electropherograms of bands 1 and 3 probably represents fragmented RNA. However, the rough membranes (bands 2 and 4) contain a considerable amount of smaller RNA molecules which could represent messengers.

These data are consistent with the hypothesis that much of the predominant species of 28S RNA could be a virion RNA precursor synthesized in the cytoplasm where it is rapidly assembled with nucleoprotein into nucleocapsids. Only later in infection do the nucleocapsids containing 28S (and 42S) RNA become associated with membrane where they presumably are enveloped and released as virions.

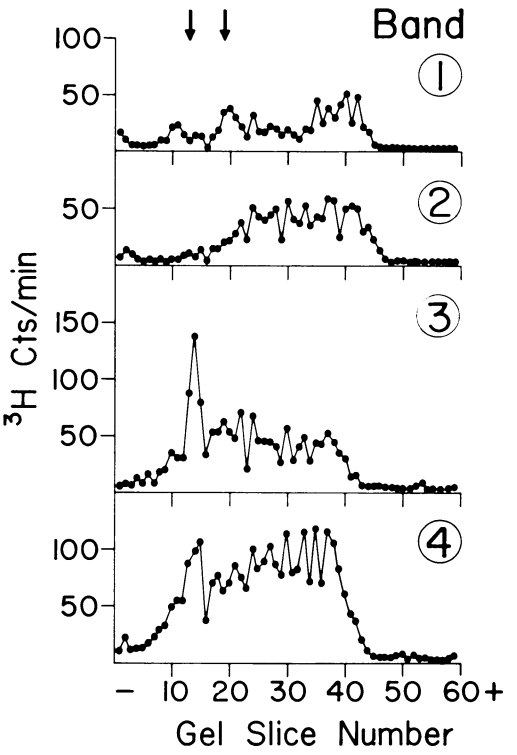

FIG. 8. *Comparative electropherograms of ^{3}H-RNA species associated with smooth membrane (band 1), rough membrane (band 2), nucleocapsid fraction (band 3), and microsomal fraction (band 4) of 12.6 × 10^7 L cells infected with VS virus for 2.5 hr and pulse labeled for 10 min with ^{3}H-uridine (25 μCi/ml). After labeling, the cells were washed twice with cold BSS, scraped into BSS, pelleted, and suspended in 3.5 ml of RSB. After allowing the cells to swell for 20 min, they were disrupted by 20 strokes of a tight-fitting Dounce homogenizer. The nuclei were removed at 800 × g and the cytoplasmic contents were centrifuged in the type SW 27 rotor for 18 hr at 96,000 × g (see Materials and Methods). The ^{3}H-RNA from the four light-scattering bands was extracted with SDS and phenol and washed twice with cold ethanol. The viral ^{3}H-RNA was analyzed by electrophoresis for 170 min in 2.3% polyacrylamide gels along with marker ribosomal ^{14}C-RNA. Radioactivity of gel slices was determined by scintillation spectrometry; arrows indicate the peak regions of ribosomal 28S and 18S ^{14}C-RNA.*

DISCUSSION

These studies and those of Cohen et al. (6) suggest that the envelope and nucleocapsid proteins of VS virus are synthesized at separate

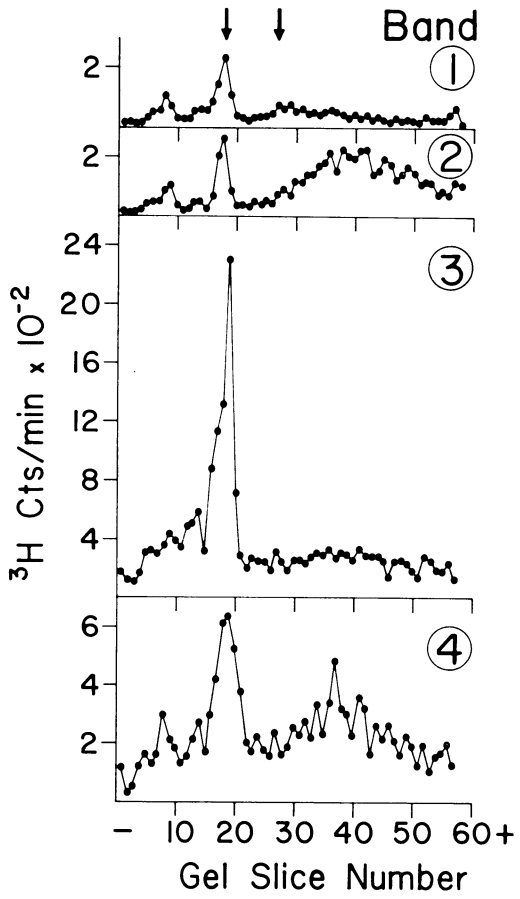

FIG. 9. *Electrophoretic profiles of the VS viral ^{3}H-RNA species associated with smooth membrane (band 1), rough membrane (band 2), nucleocapsid fraction (band 3), and rough membrane plus ribosomes (band 4) of infected L cells labeled for 90 min at 3.5 hr postinfection. The procedures for labeling with ^{3}H-uridine (10μCi/ml), fractionation of membranes, and other cytoplasmic contents on 0 to 60% discontinuous sucrose gradients and analysis of banded ^{3}H-RNA on acrylamide gels were identical to those described in Fig. 8. Arrows signify the peak regions of 28S and 18S ribosomal marker ^{14}C-RNA.*

membrane and nonmembrane sites in the cytoplasm. Consistent with this finding is the evidence that VS virus, unlike poliovirus, synthesizes short complementary RNA species which serve as messengers (9, 12, 14, 19). It seems possible, therefore, that some viral messengers could be membrane bound where they translate proteins which are inserted directly into membrane. It seems likely from previous studies with VS viral mutant *ts* 52 that glycosylation is essential for membrane insertion of the G protein (13). Other viral messengers may not be membrane associated and could function on polysomes free in the cytoplasm. Previous studies (18) indicate that protein N is made in membrane-free cytoplasm as is protein NS_1 which is present throughout infection in a soluble form. Taken together, these data indicate the existence of two cytoplasmic compartments for synthesis of two different sets of VS viral proteins which become nucleocapsid and envelope.

Our investigation of the intracellular RNA species of VS virus are not as far advanced as viral protein studies. We have not yet begun to examine the mRNA species which have to be done by hybridization studies. However, Kiley and Wagner (*unpublished data*), following the leads of other investigators (9, 12, 14, 19), have detected three species of virion RNA in infected cells; these RNA species have sedimentation coefficients of 23*S*, 28*S*, and 42*S* and are present as nucleocapsids in infected cells as well as in released virions. We have some preliminary evidence that 28*S* is the predominant species of nucleocapsid RNA and that it is linked to 23*S* RNA to make the infective 42*S* RNA-containing B virion. The studies reported here indicate that 28*S* RNA is first present in membrane-free cytoplasm and is later associated with cytoplasmic membrane. These findings are consistent with the elegant experiments of Cohen et al. (6) who demonstrated that VS viral nucleoprotein becomes associated with plasma membrane only after a long chase. It seems likely, therefore, that VS viral RNA and proteins N, L, and NS_1 are assembled into nucleocapsids in the cytoplasm and later attach to cytoplasmic membrane containing viral G and S proteins.

The major drawback in all these studies is the limited degree of confidence that one has in identification of cytoplasmic membrane components. There is little agreement among various laboratories concerning the best way of isolating cellular membranes, particularly the outer plasma membrane. The method devised by Atkinson and Summers (1) seems quite good. However, under no circumstances is there assurance of purity. We have some reservations about the Caliguiri-Tamm procedure, which we used in our studies, because they considered the membrane fraction in the top band to be smooth ER. In our experiments, we produced greater fragmentation of plasma membrane by vigorous Dounce homogenization and saw few membrane particles in the 800 × *g* nuclear pellet by phase microscopy. In addition, the presence of ouabain-sensitive Na^+, K^+-dependent adenosine triphosphatase suggests enrichment of band 1 smooth membrane with plasma membrane (2, 4).

Regardless of the exact identity of the mem-

brane components, the data available to date permit us to propose a testable hypothesis for the total replication of VS virus. The first step appears to be entry of the VS viral nucleocapsid after fusion of the virion envelope with the host cell plasma membrane (8). The next event is synthesis of mRNA catalyzed by the parental virion transcriptase (3). The mRNA species presumably attach to membrane-bound ribosomes or to cytoplasmic ribosomes and synthesize their respective membrane proteins and cytoplasmic proteins. There appears to be no regulation of protein synthesis at the level of transcription, but there may be at the translational level (18). Progeny RNA appears to be made in the cytoplasm on complementary messengers, possibly on 28*S* and 23*S* templates. The progeny RNA then assembles into cytoplasmic nucleocapsids with major proteins N and minor proteins L and NS_1. Unpublished data of Emerson and Wagner and Prevec (*personal communication*) indicate that nucleocapsids contain these three proteins as well as transcriptase activity. The nucleocapsid then attaches to the cytoplasmic membrane that has been converted to viral envelope by insertion of proteins G and S. Choppin (*personal communication*) has some evidence that the nonglycosylated (S) membrane protein may serve as the binding site of nucleocapsid to membrane. After association of the nucleocapsid and converted membrane, the virus buds.

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LITERATURE CITED

1. Atkinson, P. A., and D. F. Summers. 1971. Purification and properties of HeLa cell plasma membrane. J. Biol. Chem. **216**:5162–5175.
2. Avruch, J., and D. F. H. Wallach. 1971. Preparations and properties of plasma membrane and endoplasmic reticulum from isolated rat fat cells. Biochim. Biophys. Acta **233**:334–347.
3. Baltimore, D., A. S. Huang, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Nat. Acad. Sci. U.S.A. **66**:572–576.
4. Brunette, D. M., and J. E. Till. 1971. A rapid method for isolation of L-cell surface membranes using an aqueous two-phase polymer system. J. Memb. Biol. **5**:215–224.
5. Caliguiri, L. A., and I. Tamm. 1970. The role of cytoplasmic membranes in poliovirus biosynthesis. Virology **42**:100–122.
6. Cohen, G. H., P. H. Atkinson, and D. F. Summers. 1971. Interaction of vesicular stomatitis virus structural proteins with HeLa plasma membranes. Nature N. Biol. **231**:121–123.
7. Duesberg, P. H., and C. D. Blair. 1966. Isolation of the nucleic acid of mouse mammary tumor virus (MTV). Proc. Nat. Acad. Sci. U.S.A. **55**:1490–1497.
8. Heine, J. W., and C. A. Schnaitman. 1971. Entry of vesicular stomatitis virus into L cells. J. Virol. **8**:786–795.
9. Huang, A. S., D. Baltimore, and M. Stampfer. 1970. RNA synthesis of vesicular stomatitis virus. III. Multiple complementary messenger RNA species. Virology **42**:946–957.
10. Huang, A. S., J. W. Greenawalt, and R. R. Wagner. 1966. Defective T particules of vesicular stomatitis virus. I. Preparation, morphology, and some biologic properties. Virology **30**:161–172.
11. MacGregor, C. H., and C. A. Schnaitman. 1971. Alterations in the cytoplasmic membrane proteins of various chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **108**:564–570.
12. Mudd, J. A., and D. F. Summers. 1970. Polysomal RNA of vesicular stomatitis virus-infected HeLa cells. Virology **42**:958–968.
13. Printz, P., and R. R. Wagner. 1971. Temperature-sensitive mutants of vesicular stomatitis virus: synthesis of virus-specific proteins. J. Virol. **7**:651–662.
14. Schincariol, A. L., and A. F. Howatson. 1970. Replication of vesicular stomatitis virus. I. Viral specific RNA and nucleoprotein in infected L cells. Virology **42**:732–743.
15. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron transport system associated with the outer membrane of liver mitochondria. J. Cell Biol. **32**:415–438.
16. Wagner, R. R., J. W. Heine, G. Goldstein, and C. A. Schnaitman. 1971. Use of antiviral-antiferritin hybrid antibody for localization of viral antigens in plasma membrane. J. Virol. **7**:274–277.
17. Wagner, R. R., T. C. Schnaitman, R. M. Snyder, and C. A. Schnaitman. 1969. Protein composition of the structural components of vesicular stomatitis virus. J. Virol. **3**:611–618.
18. Wagner, R. R., R. M. Snyder, and S. Yamazaki. 1970. Proteins of vesicular stomatitis virus: kinetics and cellular sites of synthesis. J. Virol. **5**:548–558.
19. Wild, T. F. 1971. Replication of vesicular stomatitis virus: characterization of the virus-induced RNA. J. Gen. Virol. **13**:295–310.
20. Yamazaki, S., and R. R. Wagner. 1970. Action of interferon: kinetics and differential effects on viral functions. J. Virol. **6**:421–429.