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Isolation of the Envelope of Vesicular Stomatitis Virus

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Vesicular stomatitis virus was disrupted by a combination of freezing and thawing, osmotic shock, and sonic treatment. Subviral components were separated by isopycnic centrifugation. The low-density, lipid-rich fractions were pooled and shown to contain primarily viral glycoprotein. Further purification of this material resulted in the isolation of a preparation of vesicles which contained only the G protein and the same phospholipids as in the intact virions and exhibited spikelike structures similar to those on intact vesicular stomatitis virions. We conclude that we have isolated fragments of native vesicular stomatitis virus envelopes.

A series of events describing the maturation of vesicular stomatitis virus (VSV) and other enveloped RNA viruses has been postulated based on in vivo studies (5, 7, 15). The molecular mechanisms involved in assembly have not been investigated directly, since the various stages cannot be clearly isolated in the in vivo system. In vitro reconstitution systems have not been available, in part due to the inability to obtain intact viral envelopes.

VSV has a nucleocapsid core which contains RNA and the major nucleocapsid protein, N (6, 16). Two minor proteins, L and NS, are also associated with the nucleocapsid (4). The envelope of the virion appears to be a lipid bilayer derived from the host cell plasma membrane (12). A single glycoprotein species, G, is associated with the envelope and appears as spikes on the virion (12, 15). The M or matrix protein is believed to lie between the nucleocapsid core and the envelope (2, 15). Nucleocapsid cores with the ability to transcribe can be isolated (3), but until now there have been no procedures available for the isolation of intact viral envelopes.

In this paper we report the successful isolation of VSV envelopes without the use of detergents.

MATERIALS AND METHODS

Cells. BHK-21 cells and mouse L cells (clone 929) were obtained from the American Type Culture Collection (ATCC). Monolayer cultures were maintained in complete medium (MEM-C), which consisted of Eagle minimal essential medium (MEM) supplemented with 7% calf serum and antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml).

The mouse L cells were adapted to grow in suspension and be maintained as Spinner cultures in complete MEM suspension medium supplemented with 2 mM glutamine.

Virus. Our original stock of VSV Indiana (3B-Glasgow) was a gift from Robert Wagner. A heat-resistant variant was selected for the ability to survive heating for 10 min at 50°C and was cloned by three rounds of plaque isolation. This isolate was used to prepare a large stock of virus in monolayers of BHK-21 cells and was stored in portions at −80°C. All experiments were performed with virus grown directly from this seed stock.

Virus was prepared by infecting BHK-21 cell monolayers in Costar 150-cm^2 plastic flasks with 3 ml of inoculum at a multiplicity of 0.1 to 1.0 plaque-forming units (PFU)/cell. After 30 min at 37°C, 20 ml of fresh MEM-C was added, and the infection was allowed to proceed for an additional 18 to 20 h at 37°C. The culture fluid was collected and cleared of cell debris by low-speed centrifugation (1,000 × *g*) for 30 min at 4°C (clarified virus).

Virus purification. Virus was maintained at 4°C throughout the purification. Clarified virus was centrifuged for 1 h at 30,000 rpm in a Spinco 60Ti rotor. Pellets were resuspended in TSE buffer (0.05 M Tris–0.15 M NaCl–0.5 mM EDTA, pH 7.7) and pelleted again in the same way. These pellets were resuspended to 2% of the starting volume in TSE. The virus was sonically treated with a titanium microprobe on a Branson Sonifier (model W185) for two bursts of 15 s at 30 to 40 W separated by a 30-s interval and was then applied to 5 to 40% linear sucrose gradients (weight/weight solutions of sucrose prepared in TSE buffer with 5 mM MgCl replacing the EDTA; TSM). Gradients were spun for 90 min at 21,000 rpm in an SW27 rotor. The single visible band of virus was collected in a syringe by puncturing the side of the tube. The virus was diluted with TSM and sedimented in an SW27 rotor at 25,000 rpm for 90 min. The final pellet was resuspended in TSM, sonically treated as described above, and stored at −80°C.

Virus assay. Plaque assays were performed on L-cell monolayers prepared by adding 1% 1.8 M $CaCl_2$ to Spinner cultures and plating approximately 5 × 10^6 cells per 60-mm plastic petri dish. Monolayers were ready for use within 2 h after plating. The plaque overlay medium consisted of MEM containing 1%

Difco agar, 7% calf serum, 0.13% $NaHCO_3$, 50 μg of DEAE-dextran per ml, antibiotics, and 33 μg of neutral red per ml. Diluted virus (0.5 ml/dish) was allowed to adsorb for 30 min at 37°C before 3.5 ml of overlay medium was added. Cultures were incubated for 48 h at 37°C before counting the plaques.

Isotopic labeling of virus. Lipids were labeled by washing BHK-21 cell monolayers with MEM and then incubating them for 4 h at 37°C with MEM containing 1 μCi of [*methyl*-^{3}H]choline chloride per ml (3 to 5 Ci/mmol). Cells were then infected as described above and incubated in MEM-C.

To label proteins, medium was removed 2 h after infection, and fresh MEM-C containing 1 μCi of ^{14}C-amino acids (algal protein hydrolysate) per ml was added. Virus was harvested after incubation for an additional 16 to 18 h.

For virus labeled in RNA and protein, cells were infected as usual, and after 2 h, the medium was replaced with MEM-C containing 1 μCi of [5-^{3}H]uridine per ml (30 Ci/mmol) and 1 μCi of ^{14}C-amino acids per ml. Virus was harvested 16 to 18 h later.

Phospholipid analyses were performed on virus labeled with [2-^{3}H(N)]glycerol. Uninfected cells were labeled for 4 h using MEM lacking glucose and containing 10 μCi of [^{3}H]glycerol per ml (5 to 10 Ci/mmol). Infection and protein labeling were carried out as above.

Polyacrylamide gel analysis. A modification of Neville's (13) discontinuous gel system was used for both tube and slab gels. The running gel contained 9% acrylamide and 0.1% bisacrylamide, and the stacking gel contained 3% acrylamide and 0.2% bisacrylamide.

Samples containing 10 μg of ovalbumin as carrier were precipitated by the addition of an equal volume of 15% trichloroacetic acid, solubilized using the Laemmli solution (8), and boiled for 3 min before being applied to the gels.

Preparation for fluorography followed the procedure of Bonner and Laskey (1). Densitometric tracings of the fluorograms were made using the linear transport module of a GCA/McPherson EU700 digital spectrophotometer interfaced with a Hewlett-Packard 9810 A calculator/9862 A plotter recording system. The distribution of protein in the various virus bands was determined by integrating the area under the peaks of the tracings. The percent protein in a given peak was obtained by dividing by the sum of the areas under all the peaks present in the particular sample. Controls were performed to assure that all exposures were linear with respect to ^{14}C counts in a given band.

Isopycnic centrifugation. All sucrose solutions were prepared on a weight/weight basis in Tris-saline (TS) buffer (0.05 M Tris–0.15 M NaCl, pH as indicated in text) made up in deuterium oxide (D_2O). Linear 15 to 30% sucrose-D_2O gradients (9.2 ml) were formed above 0.3-ml cushions of 70% sucrose in Spinco SW41 polyallomer tubes. A 1.5-ml sample was applied to each gradient, and the tubes were spun for 3 days at 37,000 rpm in a Spinco 41 rotor. Twenty-five to 40% of the applied infectivity was routinely recovered following gradient centrifugation of intact VSV.

Phospholipid analysis. Samples precipitated with trichloroacetic acid (as described) were extracted with chloroform-methanol (2:1), and the extracts were washed with water to remove residual trichloroacetic acid and glycolipids. Extracts were dried under a stream of nitrogen and resuspended in chloroform. The preparations were then chromatographed on silica gel thin-layer plates using a solvent mixture containing chloroform-methanol-acetic acid-water in a ratio of 50:25:7:3. This system clearly separated the phospholipids, including sphingomyelin, which was not labeled but could be visualized by exposure to iodine vapor. The neutral lipids traveled with the solvent front. Five-millimeter sections of the silica gel were scraped from the plate into scintillation vials containing 10 ml of a scintillation cocktail containing toluene-Liquifluor-BioSolv (334:16:50) in addition to 0.5 ml of distilled water.

Materials. [*Methyl*-^{3}H]choline choloride, [5-^{3}H]uridine, [2-^{3}H(N)]glycerol, and Liquifluor were purchased from New England Nuclear Corp., reconstituted protein hydrolysate-algal profile-^{14}C-amino acids were purchased from Schwarz/Mann, deuterium oxide (99.8% pure) was purchased from Sigma Chemical Co., and BioSolv Solubilizer formula BBS-3 was purchased from Beckman.

RESULTS

Initial disruption of the virions. Virus labeled in both lipid and protein (approximately 10^{10} PFU/ml) was subjected to three cycles of rapid freezing and thawing in an ethanol-dry ice bath. Equal volumes of virus and 40% (wt/wt) sucrose in TSM were mixed, and the virus was pelleted by centrifugation in a Spinco SW56 rotor for 2 h at 50,000 rpm (4°C). The supernatant contained about 5% of the radioactivity and less than 0.1% of the input PFU. Polyacrylamide gel electrophoresis of the supernatant revealed all five viral polypeptides.

The tightly packed viral pellet was overlaid with cold glass-distilled water and dispersed using a sterile glass stirring rod. After incubation at 4°C for about 2 h, the remaining virus clumps were completely dispersed by repeated aspiration with a Pasteur pipette. Virus from several tubes was pooled, diluted 1:4 with water, and sonically treated in 4-ml portions. Each portion of virus was subjected to eight 15-s bursts (30 to 40 W) with 30-s intervals; virus was maintained at 4°C throughout the procedure. We found that the sonic treatment was considerably more effective when performed with diluted virus in small volumes; results were quite variable with more concentrated virus or with larger volumes. After sonic treatment, the suspension was concentrated in an Amicon ultrafiltration apparatus (Diaflo PM 10 filters; 25 mm) to the original volume. Aggregates which formed during filtration were dispersed by four 15-s bursts with the Sonifier.

The subviral components resulting from this treatment were separated by isopycnic centrifugation on sucrose-D_2O gradients at pH 7.7.

Figure 1A shows profiles of the distribution of ^{3}H-lipid and ^{14}C-protein across the gradient. Three peaks of radioactivity were seen. At the bottom of the gradient, on the 70% sucrose cushion, was a peak (peak III) which contained 10 to 15% of the labeled protein and essentially no [^{3}H]choline. A second peak (peak II) was located at approximately the same density (1.23 g/cm^{3}) as undisrupted virus in control gradients. Toward the top of the gradient, at a density of approximately 1.18 g/cm^{3}, was a third peak (peak I) which had a significantly higher lipid-protein ratio than peak II. Infectivity assays on pooled fractions (Fig. 1B) showed that 95% of the recovered infectivity appeared in peak II, whereas the protein-rich peak at the bottom of the gradient (peak III) and the lipid-rich peak near the top (peak I) contained relatively little infectivity.

Control experiments showed that none of the three individual steps, i.e. freeze-thaw, osmotic shock, or sonic treatment, was capable of disrupting the virus significantly, although each treatment alone reduced infectivity by 1 to 2 logs. Increasing the number of cycles of freezing and thawing and/or the time of sonic treatment produced no further disruption. The procedure described routinely resulted in recovery of 20 to 30% of the lipid in peak I.

Characterization of the subviral components obtained from the pH 7.7 gradient. Peak III, the material at the bottom of the gradient, appeared to be nucleocapsid. Polyacrylamide gel analyses (Fig. 2, channel b) revealed the presence of the nucleocapsid proteins L, NS, and N as well as a variable amount of M.

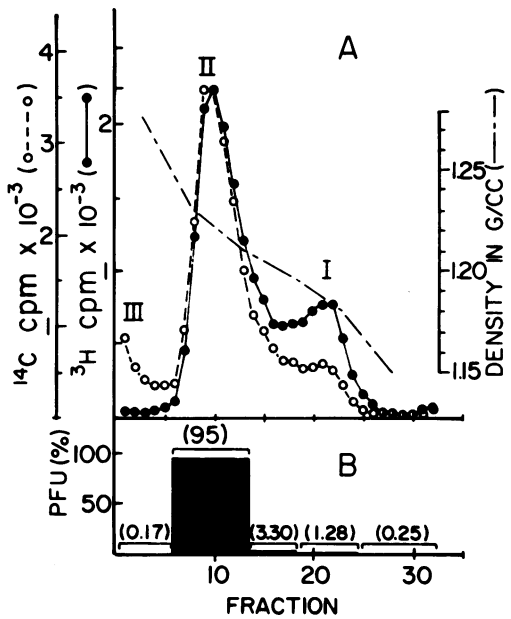

FIG. 1. *Isopycnic centrifugation of disrupted virus at pH 7.7. Purified VSV labeled with [^{3}H]choline and ^{14}C-amino acids was disrupted by a combination of freezing and thawing, osmotic shock, and sonic treatment as described and then centrifuged to equilibrium on a 15 to 30% sucrose-D_2O gradient. (A) Profiles of ^{3}H and ^{14}C obtained by liquid scintillation counting of 25-μl samples from each fraction. (B) Gradient fractions were pooled as indicated in the figure, and portions of each pool were assayed for infectivity. Percent PFU refers to the PFU in the pool expressed as percentage of the total infectivity recovered across the gradient.*

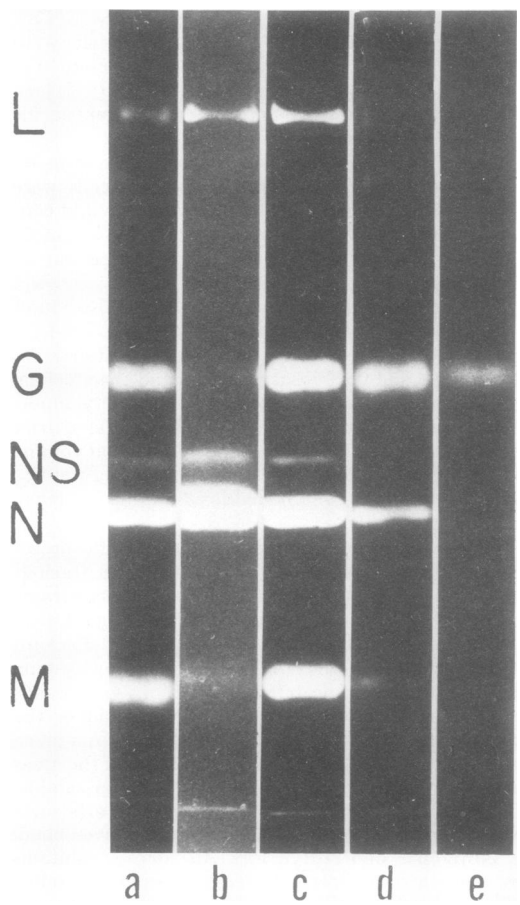

FIG. 2. *Polyacrylamide gel analyses of virion proteins. Virus was labeled with ^{14}C-amino acids, and the indicated preparations were applied to a slab gel. Prints were prepared directly from the fluorograms. (a) Purified VSV prior to treatment. (b) Pooled fractions from peak III, pH 7.7 gradient (Fig. 1). (c) Pooled fractions from peak II, pH 7.7 gradient (Fig. 1). (d) Pooled fractions from peak I, pH 7.7 gradient (Fig. 1). (e) Pooled fractions from peak Ia, pH 6 gradient (Fig. 3).*

There was essentially no G protein and no choline-labeled material, indicating the absence of viral envelope. When virus was labeled with [^{3}H]uridine and ^{14}C-amino acids and treated as described above, labeled RNA was associated only with peaks II and III. Electron micrographs of negatively stained material from peak III showed helical structures typical of nucleocapsid. There was considerable variation in the length of the pieces, and large aggregates were seen. All the evidence supports the conclusion that peak III contained nucleocapsid.

The major peak (peak II) contained undisrupted and partially disrupted virions as shown by the infectivity assays and the gel analyses in which all five peptides of the virus were present (Fig. 1B and 2c). Electron micrographs confirmed the presence of intact and broken particles.

Of greatest interest was the lipid-rich material in peak I which appeared to represent the viral envelope. Gel analyses indicated that this fraction was enriched for the viral glycoprotein, G, which accounted for approximately 65% of the total protein in peak I, although G protein comprised only about 25% of the protein in whole virions (Fig. 2d and a). The presence of small amounts of the other viral proteins was consistent with the residual infectivity associated with this peak, indicating contamination with intact and/or partially disrupted virus.

Approximately 90% of the L, G, NS, and N and 65% of the M protein of the starting virus was recovered in the three peaks.

Further purification of the envelope fraction. Pooled fractions from peak I were diluted with an equal volume of TS buffer at pH 6. This suspension was layered onto a cushion of 100% glycerol in polyallomer tubes and subjected to centrifugation at 39,000 rpm for 2 h (4°C) in the Spinco SW41 rotor. The interface was collected in a syringe after puncturing the side of the tube, diluted with TS buffer at pH 6, and sonically treated with four bursts of 15 s each. This material was subjected to isopycnic centrifugation through sucrose-D_2O gradients at pH 6.

The choice of pH 6 was made after screening a number of procedures aimed at disrupting weak, noncovalent bonds in an attempt to release trapped virions and/or weakly bound nucleocapsid fragments. These procedures included increased or decreased saline concentration, elimination of saline entirely, addition of EDTA, and variation of the pH between 6 and 9. None of these conditions successfully eliminated nonenvelope components from the isolated peak I material except pH 6. The profile of radioactivity across the pH 6 gradient is shown in Fig. 3. A prominent peak (peak Ia) was located at a density of 1.185 g/cm^3 and contained no detectable infectivity. Gel electrophoresis showed G protein and very faint bands in the areas of the N and M proteins (Fig. 2e). Densitometry showed that the G band accounted for 94% of the protein on the gel. Electron micrographs of this fraction showed vesicular structures (Fig. 4), many of which contained spikes similar in appearance to the surface spikes seen on intact VSV. The phospholipid composition of

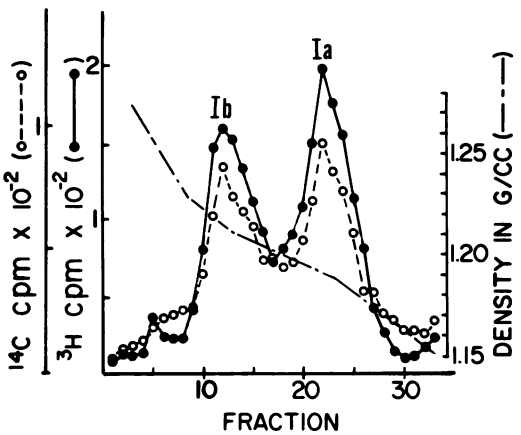

FIG. 3. *Isopycnic centrifugation of peak I. Fractions 19 to 24 from the gradient shown in Fig. 1 were pooled, diluted with TS, pH 6, and sedimented onto a 100% glycerol cushion. The interface was collected, diluted, sonically treated, and subjected to isopycnic centrifugation on a 15 to 30% sucrose-D_2O gradient at pH 6. Samples of 100 μl were counted.*

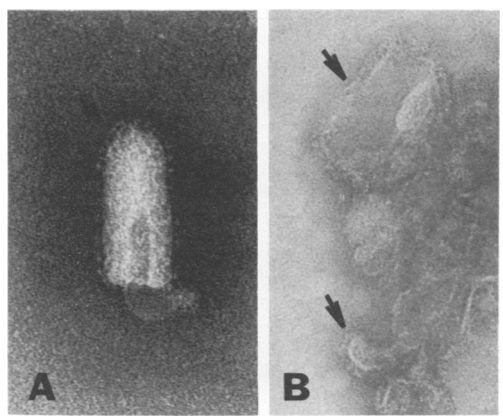

FIG. 4. *Electron micrographs of untreated VSV und the purified envelope preparation. Preparations were applied to Formvar-coated grids and stained with 2% ammonium molybdate, pH 6.5. The photographs have been adjusted to the same magnification. The arrows show the spike structures in both preparations. (A) Intact VSV. (B) Purified envelope from pooled fractions 20 to 26, Fig. 3.*

peak Ia was identical to that of the intact virion (Fig. 5). The ratio of counts per minute in ^{14}C-G protein-^{3}H-lipid was not significantly altered by the isolation procedure. This ratio in intact purified virus was 0.5, and the ratio in peak Ia material was 0.4.

A second peak (peak Ib) contained N and G proteins in a ratio of approximately 2:1, together with trace amounts of the other viral proteins.

DISCUSSION

We have disrupted VSV particles without the use of detergents and have isolated the envelope fraction. Identification of the isolated material as viral envelope was based on biochemical and morphological criteria. The similarity of the phospholipid compositions of whole virions and isolated envelopes indicated that the purification procedure did not cause selective loss of lipid components. Gel analyses of the envelope fraction showed that the G protein was the only peptide component consistent with the evidence that G is an integral protein of the viral membrane (11, 14). The constancy of the ratio of the G protein-^{3}H-lipid through the isolation procedure provides further evidence that the techniques used did not cause gross changes in the envelope structure. The spikelike projections seen in the electron micrographs suggest that the G protein is likely to be oriented in a manner similar to that in the intact virion. We conclude that these structures are indeed native fragments of VSV envelopes.

It is of interest that the M protein did not partition with the envelope fraction during the isolation procedure, whereas a significant amount of M was found with nucleocapsid. This observation agrees with studies of VSV and paramyxoviruses which showed that M protein was not solubilized by Triton under the same conditions used for release of viral glycoproteins and remained with the nucleocapsid under these conditions (3, 10). The present study indicates that if M is associated with the envelope in the intact virion, it can be dissociated without disrupting the envelope. The association of M protein with nucleocapsid is consistent with the idea that M may serve to attach the nucleocapsid to the virus-modified plasma membrane during virus assembly (10). In vitro reconstitution studies with the isolated components should permit a critical test of this assumption.

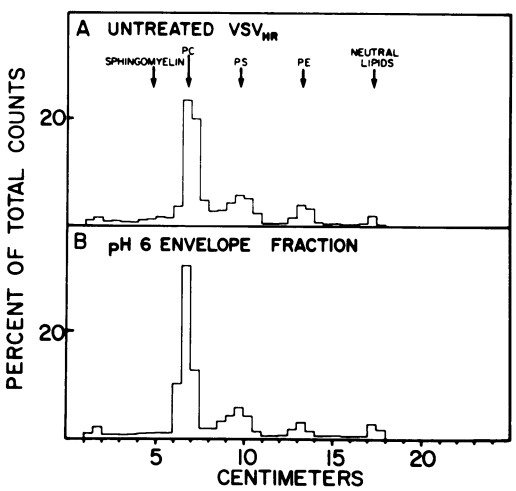

FIG. 5. *Phospholipid analyses of intact VSV and purified envelope fraction. [2-^{3}H(N)]glycerol was used to label the phospholipids, and the labeled virus was disrupted and fractionated as described. Extracts of VSV and the purified envelope fraction (pooled fractions 20 to 26, Fig. 3) were analyzed by thin-layer chromatography as described. Sphingomyelin was not labeled under these conditions, but was visualized by exposure to I_2 vapor. The identification of the different phospholipids was based on co-chromatography of unlabeled standards. PC, Phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine.*

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LITERATURE CITED

1. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
2. **Cartwright, B., P. Talbot, and F. Brown.** 1970. The proteins of biologically active sub-units of vesicular stomatitis virus. J. Gen. Virol. **7**:267–272.
3. **Emerson, S. U., and R. R. Wagner.** 1972. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. J. Virol. **10**:297–309.
4. **Emerson, S. U., and R. R. Wagner.** 1973. L protein requirement for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **12**:1325–1335.
5. **Hay, A. J.** 1974. Studies on the formation of the influenza virus envelope. Virology **60**:398–418.
6. **Kang, C. Y., and L. Prevec.** 1969. Proteins of vesicular stomatitis virus. I. Polyacrylamide gel analysis of viral antigens. J. Virol. **3**:404–413.
7. **Kang, C. Y., and L. Prevec.** 1971. Proteins of vesicular stomatitis virus. III. Intracellular synthesis and extracellular appearance of virus-specific proteins. Virology **46**:678–690.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
9. **McSharry, J. J., R. W. Compans, and P. W. Choppin.** 1971. Proteins of vesicular stomatitis virus and of phenotypically mixed vesicular stomatitis virus-simian virus 5 virions. J. Virol. **8**:722–729.
10. **McSharry, J. J., R. W. Compans, H. Lackland, and P. W. Choppin.** 1975. Isolation and characterization of

the nonglycosylated membrane protein and a nucleocapsid complex from the paramyxovirus SV5. Virology **67**:365–374.

11. **Mudd, J. A.** 1974. Glycoprotein fragment associated with vesicular stomatitis virus after proteolytic digestion. Virology **62**:573–577.
12. **Nakai, T., and A. F. Howatson.** 1968. The fine structure of vesicular stomatitis virus. Virology **35**:268–281.
13. **Neville, D. M., Jr.** 1971. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. **246**:6328–6334.
14. **Schloemer, R. H., and R. R. Wagner.** 1975. Association of vesicular stomatitis virus glycoprotein with virion membrane: characterization of the lipophilic tail fragment. J. Virol. **16**:237–249.
15. **Wagner, R. R., M. P. Kiley, R. M. Synder, and C. A. Schnaitman.** 1972. Cytoplasmic compartmentalization of the protein and ribonucleic acid species of vesicular stomatitis virus. J. Virol. **9**:672–683.
16. **Wagner, R. R., T. C. Schnaitman, R. M. Synder, and C. A. Schnaitman.** 1969. Protein composition of the structural components of vesicular stomatitis virus. J. Virol. **3**:611–618.