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Solubility of Vesicular Stomatitis Virus M Protein in the Cytosol of Infected Cells or Isolated from Virions

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The peripheral membrane M protein of vesicular stomatitis virus purified by detergent extraction of virions and ion-exchange chromatography was determined to be a monomer in the absence of detergent at high salt concentrations. Reduction of the ionic strength below 0.2 M resulted in a rapid aggregation of M protein. This self-association was reversible by the detergent Triton X-100 even in low salt. However, aggregation was not reversible by high salt concentration alone. M protein is initially synthesized as a soluble protein in the cytosol of infected cells, thus raising the question of how the solubility of M protein is maintained at physiological ionic strength. Addition of radiolabeled M protein purified from virions to unlabeled cytosol from either infected or uninfected cells inhibited the self-association reaction. Cytosolic fractions from infected or uninfected cells were equally effective at preventing the self-association of M protein. Self-association could also be prevented by an irrelevent protein such as bovine serum albumin. Sedimentation velocity analysis indicated that most of the newly synthesized M protein is monomeric, suggesting that the solubility of M protein in the cytosol is maintained by either low-affinity interaction with macromolecules in the cytosol or interaction of a small population of M-protein molecules with cytosolic components.

Many enveloped RNA viruses contain an internal matrix (M) protein as a part of the viral envelope. The M protein of vesicular stomatitis virus (VSV) appears to be associated in the virion both with envelope components and with the nucleocapsid (11, 16, 19, 21, 22, 27, 32–34). Subcellular fractionation experiments and immunolocalization experiments with VSV-infected cells have shown that M protein is synthesized on so-called free polyribosomes and is first found as a soluble protein in the cytosol before associating with membrane fractions and virions (1, 6, 14, 18, 20, 23, 25). In contrast to the solubility of newly synthesized M protein, it is widely reported that M protein purified from virions self-associates into an insoluble aggregate (3, 8, 24, 33). Biochemical and biophysical approaches to studying the properties of M protein and its interaction with other viral proteins would be greatly assisted by definition of conditions under which the solubility of M protein could be maintained. In addition, the insolubility of M protein isolated from virions raises the question of how the newly synthesized M protein remains soluble in the cytosol. Two general hypotheses could be considered. Either the M protein isolated from virions is physically different from cytosolic M protein, e.g., as a result of posttranslational modification, or else the cytosolic environment maintains the M protein in a soluble form. The first hypothesis is difficult to rule out. However, neither cytosolic nor virion M protein is modified with myristate or other fatty acids, and cytosolic M protein, like 90% of virion M protein (5), is not phosphorylated (B. J. McCreedy and D. S. Lyles, unpublished results). Evidence presented here supports the second hypothesis by showing that macromolecular components, probably proteins, of the cytosol inhibit the self-association of M protein.

Recently, attention has focused on the role of heat shock-related proteins in maintaining cytosolic precursor proteins in a soluble form before assembly at their ultimate destination such as mitochondria or plasma membranes (2, 4, 9). Similarly, it has been proposed that the VSV NS protein has a role in maintaining the solubility of the N protein before assembly into nucleocapsids (7, 26). We therefore considered that the solubility of M protein in the cytosol might be due to interaction with another viral or cellular protein. However, we were unable to detect association of M protein with any specific host or viral protein. In fact, most of the cytosolic M protein is monomeric. These results suggest that M-protein aggregation is prevented either by low-affinity interactions with cytosolic components or by interaction of a small population of M-protein molecules with host macromolecules.

To compare the solubility properties of cytosolic and virion M proteins, it was first necessary to define conditions under which M protein purified from virions would remain soluble and to systematically determine the conditions under which it would aggregate. VSV (Indiana serotype) was grown in BHK cells and purified by sucrose gradient centrifugation (17). M protein was purified from virions by a slight modification of the procedure of Zakowski et al. (33). The modified procedure still results in a highly purified M-protein preparation, as shown by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 1). Virion envelopes were solubilized with Triton X-100 in 0.25 M NaCl, and the nucleocapsids were removed by centrifugation at 110,000 × g_{avg} for 1 h (Fig. 1, lane 2). The soluble extract (lane 3) was subjected to ion-exchange chromatography on phosphocellulose (P11; Whatman, Inc., Clifton, N.J.) in which detergent, lipids, and G protein flow through (lane 5). The M protein was eluted in 0.65 M NaCl essentially free of lipids and detergent (data not shown) and appeared as a single band (lane 4), in about 30% yield. At this stage, the M protein remained soluble in 0.65 M NaCl in the absence of detergent. Removal of NaCl by dialysis before a second cycle of ion-exchange chromatography (33) resulted in aggregation of M protein. Since this step did not result in further purification of M protein, it was routinely omitted.

The hydrodynamic properties of purified M protein in 0.65 M NaCl were examined by sedimentation velocity and gel

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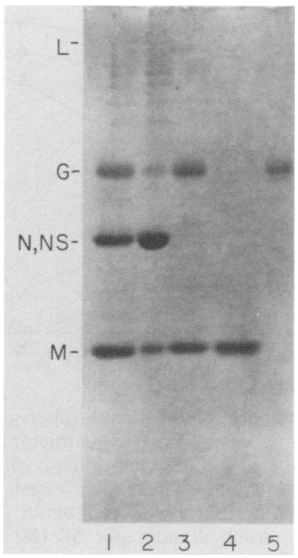

FIG. 1. Analysis of the purification of M protein by SDS-polyacrylamide gel electrophoresis. Virions were solubilized with 1% Triton X-100–0.25 M NaCl. The nucleocapsids were removed by centrifugation, and M protein was purified from the supernatant by chromatography on phosphocellulose. The different stages in the purification were adjusted to equivalent volumes (except where noted), and a 100-μl sample was analyzed by electrophoresis on a 10% polyacrylamide gel. A photograph of the gel stained with Coomassie blue R-250 is shown. Identities of the VSV proteins are indicated on the left. The N and NS proteins comigrate in this gel system. Lanes: 1, virions (diluted threefold; 15 μg of total protein); 2, nucleocapsid pellet; 3, Triton X-100 supernatant (diluted twofold); 4, M-protein fraction after ion-exchange chromatography; 5, flowthrough fraction from ion-exchange column (diluted approximately threefold).

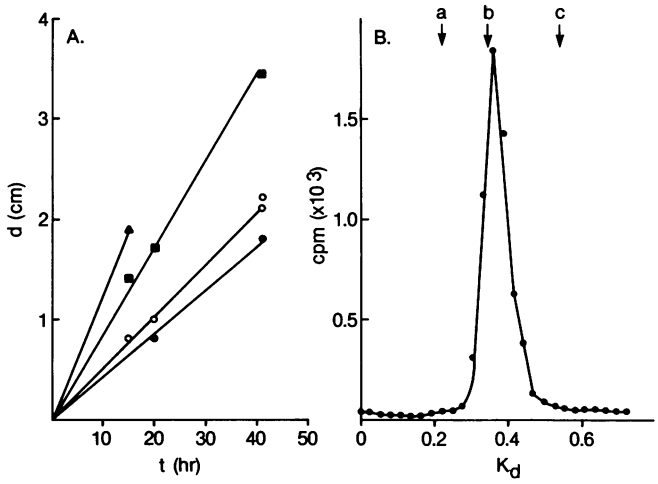

FIG. 2. Sedimentation velocity and gel filtration analysis of M protein purified from virions. (A) Sedimentation distance of M protein and standard proteins in 5 to 20% sucrose gradients containing 0.65 M NaCl as a function of time at 220,000 × *g*. Standard proteins of known $s_{20,w}$ were bovine serum albumin (▲), 4.3*s*; ovalbumin (■), 3.7*s*; and cytochrome *c* (●), 1.9*s*. The apparent $s_{20,w}$ of M protein (○), 2.0, was determined from a plot of the slopes of the above lines versus $s_{20,w}$. (B) Gel filtration of M protein on Sephadex G-150 in 0.65 M NaCl. M protein labeled with [^{3}H]leucine was chromatographed as described in the text. Radioactivity of a sample of each fraction is shown as a function of its retained fraction (K_d) of the column (void volume K_d = 0; inclusion volume K_d = 1). Standard proteins chromatographed on the same column were bovine serum albumin (a), ovalbumin (b), and soybean trypsin inhibitor (c).

filtration to determine whether purified M protein is oligomeric (Fig. 2). M protein was purified from VSV grown in the presence of [^{3}H]leucine. A 0.1-ml sample of labeled M protein (about 5 μg) was layered onto a 4.9-ml linear 5 to 20% (wt/wt) sucrose gradient containing 0.65 M NaCl–10 mM sodium phosphate (pH 7.4) and spun at 49,000 rpm for varying times in an SW50.1 rotor (220,000 × g_{avg}). The gradients were collected into 25 fractions and assayed by liquid scintillation counting. Bovine serum albumin, ovalbumin, and cytochrome *c* were centrifuged in parallel gradients and used as standards for sedimentation velocity. Physical data for standard proteins were obtained from Sober (31). Approximately 80 to 90% of freshly purified M protein sedimented as a single peak with an apparent $s_{20,w}$ of 2.0 by comparison with the standards. The remaining 10 to 20% of the M protein was found as a pellet. The fraction of M protein sedimenting as an aggregate tended to increase upon prolonged storage at 4°C or freezing and thawing.

The sedimentation velocity of M protein, which was similar to that of cytochrome *c* (molecular weight, 12,500), was slower than expected of a spherical protein of molecular weight 26,000. This appeared to be due to an elongated conformation of M protein. The Stokes radius of M protein is 2.8 nm, as determined by gel filtration in 0.65 M NaCl (Fig. 2B), similar to that of ovalbumin (molecular weight, 43,500). Assuming a typical partial specific volume for protein (0.73 ml/g), the molecular weight calculated from sedimentation and gel filtration (30) is 23,500, in good agreement with the monomer molecular weight. The calculated frictional ratio (f/f_0 = 1.48) is indicative of a moderately elongated structure.

Upon lowering of the salt concentration below 0.2 M by either dialysis or dilution, purified M protein sedimented as large aggregates (larger than decamer). Even at intermediate salt concentrations, only monomers and large aggregates were found; intermediate aggregation states (dimer, trimer, etc.) were not observed. Therefore, the fraction of M protein remaining as a monomer could be readily quantitated by pelleting the aggregates in an ultracentrifuge. This was conveniently analyzed by spinning a 120-μl sample of ^{3}H-labeled M protein in an airfuge (Beckman Instruments, Inc., Fullerton, Calif.) at 20 lb/in^2 (approximately 100,000 × *g*) and then determining the radioactivity of the supernatant and pellet by liquid scintillation counting. The kinetics of aggregation were rapid. Upon dilution to 65 mM NaCl, aggregation was complete as soon as it could be measured (10 min) even at protein concentrations as low as 5 μg/ml. The aggregation was largely reversible under the original conditions for solubilization of M protein from virions (Table 1). Unexpectedly, the active component that resolubilized M protein was not the NaCl but rather the Triton X-100. The aggregation was not reversible with 0.65 M NaCl alone. However, Triton X-100 solubilized the majority of M protein even in the absence of NaCl. Similarly, dialysis of purified, monomeric M protein to remove NaCl in the presence of Triton X-100 completely prevented the aggregation reaction (not shown). The presence of dithiothreitol, which is present in the initial solubilization of M protein from virions, is not required for resolubilization of aggregated, purified M protein. It was also noted that the aggregation of M protein that occurred upon prolonged storage or freezing and thawing was usually not reversible under the original conditions for

TABLE 1. Reversibility of M-protein aggregation[a]

NaCl concn (M)	Triton X-100 (1%)	DTT (0.2 mg/ml)	% Monomer (mean ± SD)
No addition			14.2 ± 1.9
0.65	+	+	80.4 ± 10.3
	+	−	77.2 ± 13.3
	−	+	22.6 ± 8.2
0.25	+	+	81.5 ± 13.3
	+	−	84.3 ± 8.3
	−	+	14.4 ± 2.7
0	+	+	68.6 ± 15.1

[a] Purified M protein labeled with [^{3}H]leucine (approximately 50 μg/ml) was dialyzed overnight versus 500 volumes of 10 mM sodium phosphate, pH 7.4. Samples of 120 μl were brought to the indicated final concentration of NaCl, Triton X-100, or dithiothreitol (DTT) by addition of 10-fold-concentrated stock solutions. Samples were incubated for 10 min at room temperature and then centrifuged for 10 min in a Beckman airfuge to pellet the aggregates. Radioactivity in the supernatants and pellets was determined and used to calculate the percent monomer. Data are from three separate experiments.

solubilization and therefore differed in character from the aggregation in low-salt buffers.

M protein purified from virions was not soluble at physiological ionic strength (Table 1). Purified, labeled virion M protein was mixed with the cytosolic fraction from unlabeled cells to determine whether components of the cytosol inhibit the self-association of virion M protein. Cytosolic fractions were prepared from BHK cells that were either mock infected or infected with VSV at a multiplicity of 10 to 30 PFU per cell for 4 h. Cells were washed with ice-cold 0.9% NaCl, suspended in 10 mM Tris–10 mM NaCl–1.5 mM $MgCl_2$ (pH 7.4) containing 100 kallikrein units of aprotinin (Sigma Chemical Co., St. Louis, Mo.) per ml, and allowed to swell for 20 min on ice before lysis by Dounce homogenization. Nuclei were removed by centrifugation at low speed, followed by removal of cytoplasmic membranes by centrifugation at 110,000 × g_{avg} for 45 min at 4°C. M protein, purified from virions labeled by growth in [^{35}S]methionine, was mixed with unlabeled cytosol, and the fraction remaining monomeric was determined by pelleting the aggregates, followed by liquid scintillation counting. The cytosolic fraction from uninfected cells inhibited the self-association of M protein at low ionic strength (Fig. 3). In further experiments (not shown), it was determined that the inhibitory activity was not removed by dialysis of the cytosol before the addition of M protein. Thus, the inhibitory activity had a high molecular weight. We attempted to detect a specific association between M protein and cytosolic proteins by immunoprecipitation of labeled cytosol with antibodies against M protein, followed by SDS gel electrophoresis. No protein other than M protein was precipitated above the background nonspecific level (data not shown). These experiments were performed by using cytosol labeled both before and after infection so that both host and viral proteins were labeled and were performed under conditions in which the complex of the N and NS proteins was readily detected. These negative results led us to consider that nonspecific interactions with cytosolic components might prevent the self-association of M protein. This hypothesis was supported by the observation that an irrelevant protein such as bovine serum albumin could prevent the aggregation of M protein purified from virions when added at concentrations similar to the protein concentration of the cytosolic fraction (Fig. 3). Thus, it appears likely that the cytosolic M protein may be maintained in a soluble state, at least in part, by interaction

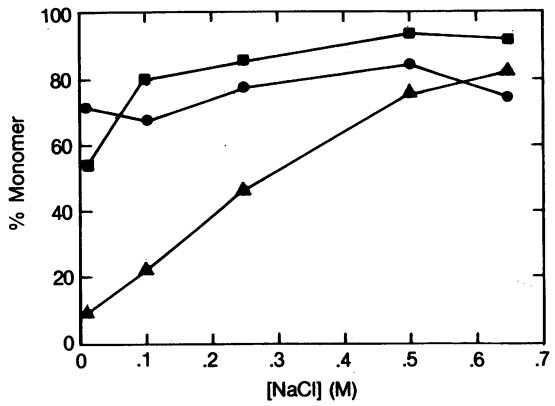

FIG. 3. Influence of cell cytosol on the aggregation of M protein. M protein (50 μg/ml) purified from [^{35}S]methionine-labeled virions in buffer containing 0.65 M NaCl was diluted with either cell lysis buffer (▲), cytosolic fraction from uninfected cells at a protein concentration of 750 μg/ml (●), or lysis buffer supplemented with 750 μg of bovine serum albumin per ml (■) to the final NaCl concentration indicated. Aggregates formed were pelleted by centrifugation in a Beckman airfuge, and the supernatants and pellets were analyzed by liquid scintillation counting.

with any of a number of high-molecular-weight components in the intracellular environment and not through interaction with specific cytosolic proteins.

The ability of the cytosolic fraction from infected cells to inhibit the self-association of M protein was compared with that of uninfected cells. M protein purified from virions grown in the presence of [^{35}S]methionine was mixed with various concentrations of cytosol from infected or uninfected cells at an NaCl concentration of 0.1 M. The percentage of M protein remaining monomeric was determined by pelleting the aggregates, followed by liquid scintillation counting. Cytosolic fractions from infected or uninfected cells were equally effective at preventing the self-association of M protein (Fig. 4).

Despite the inhibitory effect of cytosolic components on M-protein aggregation, the majority of cytosolic M protein was not tightly associated with other macromolecular species. This was shown by sedimentation velocity analysis of newly synthesized cytosolic M protein from either BHK cells or Friend erythroleukemia cells. Similar results were obtained with the two cell types. VSV-infected cells were labeled with [^{35}S]methionine (25 μCi/ml; >800 Ci/mmol; in methionine-deficient medium) for 15 min at 4 h postinfection. The cells were lysed by Dounce homogenization as described above. A portion of the homogenate was brought to 0.1 M NaCl by addition of a small volume of concentrated NaCl, and a cytosolic fraction was prepared by high-speed centrifugation. A 100-μl sample of the resulting supernatant was layered onto 5 to 20% sucrose gradients containing either 0.1 or 0.01 M NaCl and centrifuged at 49,000 rpm for 24 h in an SW50.1 rotor. The gradients were fractionated, and the fractions were analyzed by SDS gel electrophoresis and fluorography, using preexposed Kodak SB-5 film (15). M protein was quantitated by soft-laser scanning densitometry with digital integration (Biomed Instruments Inc., Fullerton, Calif.). For comparison, labeled M protein purified from virions was centrifuged in gradients containing either 0.65 or 0.2 M NaCl. The sedimentation of cytochrome *c* in parallel gradients is indicated in Fig. 5 (arrows), since the buoyant density of proteins in gradients containing 0.65 M NaCl (Fig. 5A) was significantly lower than in those containing less

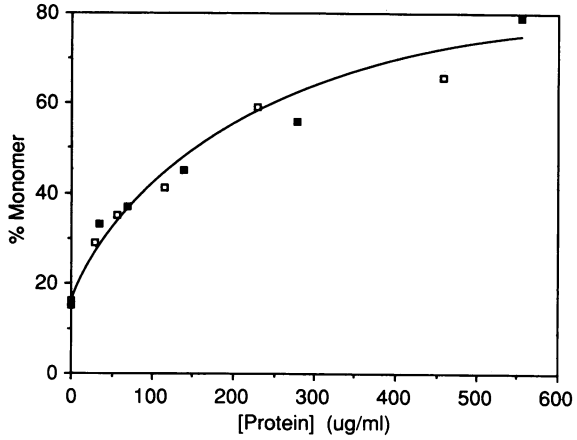

FIG. 4. Inhibition of M-protein aggregation by cytosol from infected versus uninfected cells. M protein (approximately 50 μg/ml) purified from [^{35}S]methionine-labeled virions in buffer containing 0.65 M NaCl was diluted in 10 mM Tris (pH 7.4) containing cytosol from infected (■) or uninfected (□) cells at the indicated protein concentrations. The final NaCl concentration was 0.1 M. Aggregates formed were pelleted by centrifugation in a Beckman airfuge, and the supernatants and pellets were analyzed by liquid scintillation counting.

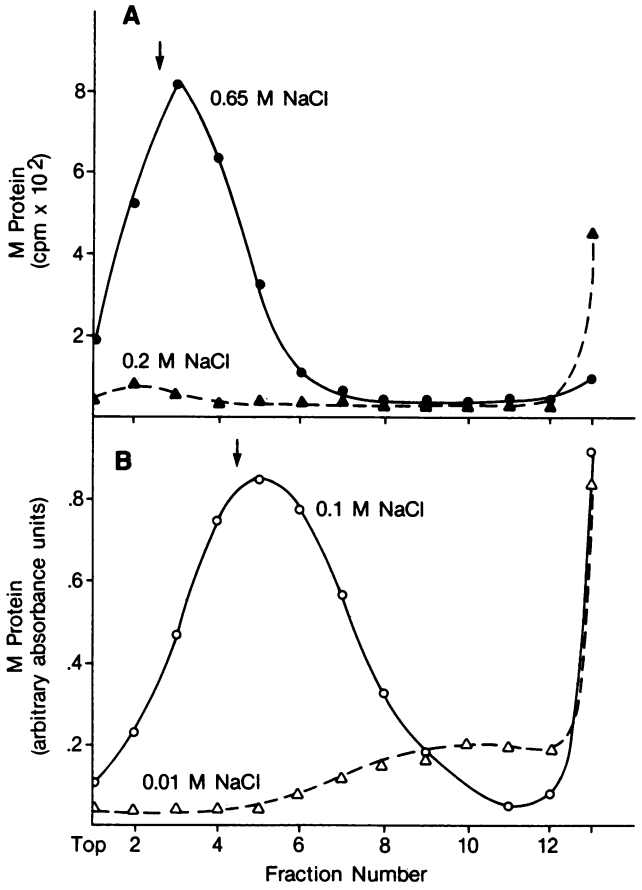

FIG. 5. Sedimentation velocity analysis of virion and cytosolic M protein. M protein purified from [^{35}S]methionine-labeled virions (A) or the cytosolic fraction from infected cells labeled with [^{35}S]methionine (B) was centrifuged at 220,000 × g for 24 h in 5 to 20% sucrose gradients containing the indicated concentrations of NaCl. After centrifugation, the gradients were fractionated, and each fraction was analyzed either by scintillation counting (A) or by SDS gel electrophoresis, fluorography, and densitometry of the M-protein band in the fluorographs (B). The arrow marks the position of cytochrome c, which was run in parallel gradients containing NaCl at a concentration of 0.65 M (A) or 0.1 M (B).

NaCl (Fig. 5B). In contrast to purified M protein, which sedimented as an aggregate in 0.2 M NaCl, sedimentation in 0.1 M NaCl of most of the cytosolic M protein (approximately 80%) was consistent with the $s_{20,w}$ of a monomer (2 compared with the sedimentation of cytochrome c). This result indicates that most of the M protein was not associated with cytosolic components large enough to affect its sedimentation velocity. Centrifugation of cytosolic M protein through gradients containing 0.01 M NaCl did result in aggregation of most of the M protein. Thus, the aggregation of cytosolic M protein was not prevented completely but was shifted to lower ionic strength.

The inhibition of M protein aggregation by cytosolic components was not due to a high-affinity interaction of the majority of M-protein molecules with cytosolic macromolecules (Fig. 5). Otherwise, the sedimentation rate of cytosolic M protein would have been greater than that of the purified protein as a result of binding to other cytosolic components. We had originally considered that cytosolic proteins related to heat shock proteins might be involved in maintaining the solubility of M protein because of their demonstrated role in the assembly of cytosolic precursors to other membrane proteins (2, 4, 9). Although our experiments do not completely rule out such a possibility, they make it very unlikely, since we analyzed the cytosolic M protein under much milder conditions than have been used to demonstrate the association of heat shock-related proteins with their targets. The interaction of M protein with cytosolic components may be of sufficiently low affinity that dissociation occurs under the conditions of centrifugation. Alternatively, the inhibition of self-association may occur by interaction of host components with a small subpopulation of M-protein molecules that are rate limiting for the self-association reaction. For example, the initiation of M-protein aggregation probably depends on the formation of some type of nucleation site, after which the reaction goes rapidly to completion, analogous to the aggregation reactions of cytoskeletal proteins such as actin (28). The nature of the nucleation event may be a conformational change in a subpopulation of M-protein molecules or formation of oligomers, among many other possibilities. Thus, the inhibition of aggregation in cytosol could occur by binding of proteins to such nucleating forms of M protein, while the bulk of the M protein remains monomeric.

The results presented here should also benefit biochemical studies on the properties of the VSV M protein by defining conditions under which the purified protein will remain soluble. This information was necessary in our study to interpret the sedimentation behavior of cytosolic M protein (Fig. 5) and to be able to perform the mixing experiments demonstrating the solubility of virion M protein in cytosol (Fig. 3 and 4). The solubility of virion M protein in detergents at high salt concentrations has been known for many years, but the solubility of the purified protein in Triton X-100 at low salt concentrations was unexpected. This result suggests that the aggregation may involve hydrophobic interactions between M-protein molecules. Even though the amino acid sequence of M protein does not include any long hydrophobic stretches (29), the folding of M protein may

generate hydrophobic surfaces involved in interaction with membranes, with the nucleocapsid, or between M-protein molecules in the virion. Prevention of such a hydrophobic interaction may also account for the ability of serum albumin or cytosolic components to inhibit M-protein aggregation. While this work was in progress, Faaberg and Peeples (12) described the inhibition of aggregation of the M protein of Newcastle disease virus by serum albumin. However, the paramyxovirus M protein appeared to form a complex with serum albumin, while the majority of the VSV M protein was monomeric. Similarly, the M protein of Sendai virus has been reported to be a dimer when solubilized in high salt (13), while that of VSV is a monomer. Thus, the biochemical properties of the M proteins of paramyxoviruses and VSV differ considerably, and these differences are reflected in their behavior in infected cells (10). Finally, we observed during the course of these experiments that aggregation of freshly prepared purified M protein is reversible, whereas aggregation that occurs upon prolonged storage is not. Thus, even though M-protein aggregation is not necessarily indicative of denaturation, studies using M-protein preparations in which the association state of the starting material is not characterized should perhaps be viewed with caution.

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