

RECONSTITUTION OF SEMLIKI FOREST VIRUS MEMBRANE

ARI HELENIUS, ERIK FRIES, and JÜRGEN KARTENBECK

From the European Molecular Biology Laboratory, Heidelberg, and the Department of Membrane Biology and Biochemistry, Institute of Experimental Pathology, German Cancer Research Center, Heidelberg, Federal Republic of Germany

ABSTRACT

The spike glycoproteins of the Semliki forest virus membrane have been incorporated into vesicular phospholipid bilayers by a detergent-dialysis method. The detergent used was β -D-octylglucoside which is nonionic and has an exceptionally high critical micellar concentration which facilitates rapid removal by dialysis. The vesicles obtained were of varying sizes and had spikes on their surface. Two classes of vesicles were preferentially formed, small protein-rich and large lipid-rich (average lipid to protein weight ratios, 0.22 and 3.5, respectively). Both classes of vesicles retained the hemagglutinating activity of the virus. The proteins were attached to the lipid bilayer by hydrophobic peptide segments, as in the viral membrane. Most of the proteins were accessible to proteolytic digestion from the outside, suggesting an asymmetric orientation.

KEY WORDS membrane biochemistry · reconstitution · Semliki forest virus · spike proteins · nonionic detergent

We are studying the Semliki forest virus (SFV) as a model membrane system. The SFV consists of a spherical nucleocapsid surrounded by a membrane (26, 30, 33). The nucleocapsid is composed of an RNA molecule (4×10^6 mol wt, [26, 34]) and one protein species (3.4×10^4 mol wt, [9, 29]). Three glycopolypeptides are found in the membrane: E1, E2, and E3, with molecular weights of 5×10^4 , 5×10^4 , and 1×10^4 , respectively (10). They form 7–8-nm long spikes projecting from the surface of the virus, and are necessary for the viral infectivity. E1 and E2 have hydrophobic peptide segments which anchor them to the membrane (38).

We have previously studied the mechanism by which two mild detergents (Triton X-100 and sodium deoxycholate, DOC) and a denaturing detergent (sodium dodecyl sulfate, SDS) dissociate the SFV membrane (5, 13, 14). With in-

creasing concentrations of these detergents the dissociation proceeded through four stages: initial binding of the detergent to the membrane, lysis, disintegration into lipid-protein-detergent and lipid-detergent complexes, and finally, delipidation of the proteins. The end products were soluble detergent-protein complexes and lipid-detergent mixed micelles. The essential difference in the action of the three detergents was in their effect on protein structure. SDS and DOC dissociated the spike proteins, whereas in Triton X-100, E1, E2, and E3 remained in the form of a complex.

After these detailed dissociation studies we have proceeded to investigate the reverse process, the reconstitution of the membrane (and the whole virus) from its isolated components. Artificial assembly of viral membrane components has been previously described by Hosaka and Shimizu (15, 16), who used the nonionic detergent Nonidet P-40 (*p*-*t*-octylphenylpolyoxyethylene₉, [Shell Chemical Co., New York.]) and dialysis to reconstitute Sendai virus membranes, and by Almeida

et al. (2), who cosonicated influenza membrane proteins with a mixture of lecithin and dicytyl phosphate. We have tested these and other methods described for membrane reconstitution (18, 23, 36). Our first approach was to isolate the membrane proteins from SFV as lipid- and detergent-free soluble octameric complexes (12), and to sonicate them with phospholipid liposomes (32). We found that the protein associated with the lipid, but the resulting vesicles tended to aggregate and were unsuitable for further study. Detergent-dialysis and detergent-dilution procedures proved more successful. We tried several bile salts and nonionic detergents, and although conditions could be found in which lipid and protein formed vesicular membranes, the results were unsatisfactory, due to vesicle aggregation (sodium deoxycholate and cholate), high contents of residual detergent (Triton X-100, *p*-*t*-octylphenylpolyoxyethylene _{$\bar{m}=9.7$}), protein inactivation (dodecyltrimethylammoniumoxide), or low yields (sodium taurodeoxycholate). Only by using β -D-octylglucoside, a nonionic detergent recently introduced into membrane studies (31, 19, 37), could we obtain satisfactory results. In this paper, we describe the reconstitution procedure and the initial characterization of the membrane structures obtained using this detergent.

MATERIALS AND METHODS

Virus and Materials

A prototype strain of SFV was grown in monolayer cultures of BHK-21 cells, purified from the cell culture medium, and analyzed for purity as described by Kääriäinen et al. (17). Virus protein was determined by the Lowry method with 3.5 mM SDS in the reaction mixture. These determinations agreed within 10% with values obtained by amino acid analysis. Radioactively labeled virus was grown and isolated as previously described (13, 17). The egg lecithin (Type V-E) was purchased from Sigma Chemical Co., St. Louis, Mo. Both preparations were 98% pure, as judged by thin layer chromatography (chloroform:methanol:4 N ammonium hydroxide vol/vol). The phospholipids were assayed as inorganic phosphorus (4). The [³²P]phospholipids were extracted from BHK-21 cells grown for 19 h in Eagle's Minimum Essential Medium containing one-tenth of the original phosphate, 10% calf-serum, and 3 mCi carrier-free [³²P]orthophosphate (The Radiochemical Centre, Amersham, Eng.). The labeled cells were harvested after treatment with trypsin-EDTA, and the lipids were extracted by the method of Folch et al. (8). Thin-layer chromatography followed by autoradiography and optical scanning showed that lecithin

accounted for 55% of the radioactivity. Phosphorus analysis gave a specific activity of 3×10^{14} dpm/mole phosphorus. The radioactive lipids were stored in the extraction solution at 0°C and used within 3 wk.

β -Octylglucoside and [¹⁴C] β -octylglucoside were synthesized according to the method of Noller and Rockwell (25). The acetobromo- α -D-glucose, used in the [¹⁴C]octylglucoside synthesis, was prepared from [¹⁴C]glucose (20). The octylglucosides were >97% pure as judged from thin-layer chromatography (benzene, acetone, methanol, 4:2:1 vol/vol). In preliminary tests we used a sample of octylglucoside, generously supplied by Dr. Carl Baron. Sodium deoxycholate (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), and sodium dodecyl sulfate (Serva, Heidelberg, Federal Republic of Germany) were used without prior purification. The ring-labeled [³H]Triton X-100 (0.21 mCi/g) was a gift from Dr. W. R. Lyman (Rohm & Haas). Thermolysin and dithiothreitol were purchased from Calbiochem, San Diego, Calif. The buffer salts were of analytical grade from Merck AG, Darmstadt, Federal Republic of Germany.

Protein Isolation and Reconstitution

A mixture of SFV (0.8 mg protein), [³⁵S]methionine, and [³²P]phosphate-labeled virus was solubilized at 22–25°C in 0.2 ml 0.025 M Tris-HCl, pH 7.4, containing 0.05 M NaCl, 20 mg/ml Triton X-100, and 8% (wt/wt) sucrose. The solubilized virus was layered onto a sucrose gradient consisting of three zones: on top a 0.4-ml 13% (wt/wt) sucrose zone with 0.5 mg/ml Triton X-100, in the middle a 3.8-ml linear 18–25% sucrose gradient containing 30 mM octylglucoside, and at the bottom a 0.5-ml 60% sucrose cushion containing 30 mM octylglucoside. All zones contained 0.1 M NaCl and 0.05 M Tris-HCl, pH 7.4. After the centrifugation (24 h, 4°C, $190 \cdot 10^3 \times g$, SW 50.1 Beckman rotor), fractions were collected from the bottom and 0.01 ml aliquots were assayed for radioactivity (Fig. 1). The [³⁵S]methionine peak containing the membrane proteins was pooled and used as such for the reconstitution. An appropriate amount of egg lecithin (75–200 μ g) in chloroform:methanol 2:1 (vol/vol), an aliquot of the ³²P-lipid extract of the BHK-cells (<2 μ g lipid), and octylglucoside in acetone (four times the amount of the lipid) were mixed in a glass tube. The solvents were removed under a stream of nitrogen and the residue was twice dissolved in 0.1 ml diethyl ether and dried. The resulting thin lipid-detergent film was then dissolved in a 1–3 ml aliquot of the isolated membrane protein. The resulting clear solution was dialyzed at 23–27°C for 36 h against 3 changes of 0.8 liter 4 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 0.1 mM dithiothreitol, and 2 mM sodium azide. The first dialysis solution also contained 0.3 M sucrose. An increase in ionic strength to 0.13, omission of MgCl₂, dithiothreitol, sucrose, or a reduction

of temperature to 7°C had little effect on the final results. Finally, the dialysate was concentrated within the bag to 0.7–0.8 ml using dry Biogel P-200 gel beads (Bio-Rad Laboratories, Richmond, Calif.).

Electron Microscopy

The reconstituted membranes were centrifuged for 90 min at 4°C and $150 \cdot 10^3 \times g$ using the Beckman SW 50.1 rotor and 0.6 ml tubes. At least 90% of the protein and 80% of the lipid was in the pellet. The pellet was fixed (at 4°C or at room temperature) for 20–40 min in 2.5% glutaraldehyde containing 0.05 M sodium cacodylate, pH 7.2, 50 mM KCl, and 2.5 mM $MgCl_2$. The fixed pellet was washed repeatedly with cacodylate buffer, postfixed for 2–3 h using 2% osmium tetroxide, washed again with distilled water, and soaked for 10–14 h in aqueous uranyl acetate (1%). All steps were performed in the cold (0°–5°C). After dehydration in graded ethanol solutions and transfer to propylene oxide, the material was embedded in Epon 812, ultrathin sectioned, and double stained with uranyl acetate (5% methanolic) and lead citrate (27, 39).

For negative staining, a drop of the sample solution was put onto a Formvar-coated copper grid, and either drawn off totally before the staining procedure so that the sample air dried on the film, or a small residue of the suspension was left on the grid to which the negative stain (2% phosphotungstic acid, adjusted with NaOH to pH 7.2) was then added. After 5–15 s, the excess stain was removed and the grids air dried.

Ultrathin section and negative stain preparations were examined with a Siemens Elmiskop 101 electron microscope. Morphometric measurements were made by projecting electron micrographs onto a digitizer tablet (MOP-AM-01; Kontron Eching-München, Federal Republic of Germany) which was connected to a computer unit (Summagraphics, Fairfield, Conn.).

Thermolysin Treatment of Reconstituted Vesicles

The three pools from the sucrose density gradient (Fig. 5) were dialyzed overnight against 0.05 M Tris-HCl, pH 7.4, containing 0.1 M NaCl at 4°C, and the volume was reduced to 0.3–0.4 ml by vacuum dialysis in a collodium sack. Thermolysin and $CaCl_2$ were added to give final concentrations of 1 mg/ml and 5 mM, respectively. The mixtures were incubated for 90 min at 37°C, and the digestion terminated by addition of EDTA (final concentration 4 mM). Solid sucrose was then added to give 60% wt/wt, and the solutions were transferred to 5-ml centrifuge tubes. The samples were overlaid with 3 ml 40% wt/wt sucrose and 1.5 ml of the Tris-buffer. After 10 h centrifugation at $190 \cdot 10^3 \times g$ at 4°C, 0.25-ml fractions were collected. The fractions containing the ^{32}P -activity were pooled, dialyzed against 0.1 M $NaHCO_3$, and freeze-dried. The residues were dissolved in 0.05 ml 10 mg/ml SDS and 20 mg/ml 2-

mercaptoethanol, and heated to 100°C for 5 min. Electrophoresis in 1.0 mg/ml SDS was then performed as described by Utermann and Simons, using 15% polyacrylamide gels (38).

Other Methods

The E1 polypeptide was isolated by centrifuging SFV into a sucrose gradient containing sodium deoxycholate, as previously described (14). The deoxycholate in the isolated E1 was then exchanged to octylglucoside by repeated dilution (with 0.05 M Tris, pH 8.5, containing 0.1 M NaCl and 30 mM octylglucoside), and concentration in a 10-ml Amicon ultrafiltration cell (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) equipped with a PM 10 membrane. Reconstitution was thereafter performed with octylglucoside, as described. Sucrose concentrations were determined by refractometry. Electrophoresis in slabs of polyacrylamide gel in the presence of SDS was performed according to Neville (24). Fluorography of the slabs was done according to Laskey and Mills (22). Radioactivity was measured in Triton X-100 toluol scintillant using a Nuclear Chicago Mark III scintillation counter (Searle Analytic Inc., Des Plaines, Ill.). Hemagglutination was assayed according to Clarke and Casals (7) using fresh goose red blood cells.

RESULTS

Isolation of the SFV-Membrane Protein in Octylglucoside

Because octylglucoside dissociates only part of the spikes from the viral nucleocapsids (unpublished observations), we used Triton X-100 for the initial solubilization (13). The Triton X-100 solubilizate was centrifuged into a sucrose gradient containing octylglucoside, and the spike polypeptides, E1, E2, and E3, were recovered in the octylglucoside-solubilized form as a broad band in the middle of the gradient (Figs. 1, 2a). The nucleocapsid protein and the RNA were found in the pellet (Fig. 2a), whereas the phospholipids and the Triton X-100 were found in the top fractions of the gradient (Fig. 1). The spike protein band contained $<10 \mu g$ phospholipid and $20 \mu g$ Triton X-100/mg protein. Electron microscopy showed that the spike proteins occurred as rosette-like aggregates with a diameter of ~ 18 nm (Fig. 3b).

Reconstitution

Egg lecithin, [^{32}P]BHK-cell lipid, and octylglucoside in the form of a dry film were dissolved in the solution containing the spike proteins, and the octylglucoside was removed by dialysis. The

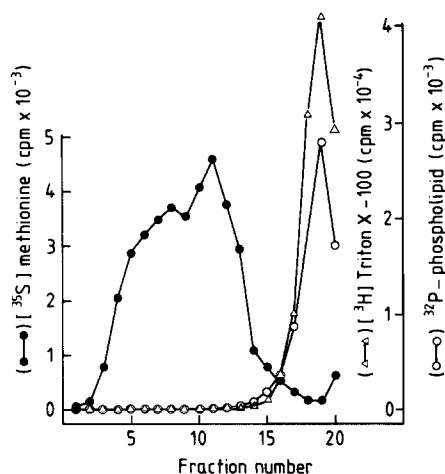


FIGURE 1 Isolation of the spike glycoproteins by sucrose gradient centrifugation. The gradient contained 30 mM octylglucoside and the sample 0.8 mg SFV solubilized with Triton X-100 (for details see Materials and Methods). The peak fractions containing [³⁵S]methionine-labeled protein were pooled and the pellet ([³⁵S]methionine and [³²P]-activities $13 \cdot 10^3$ and $7 \cdot 10^3$ cpm, respectively) solubilized with 10 mg/ml SDS. Bottom to the left.

dialysate was recovered as a slightly opalescent solution containing 80–90% of the protein and lipid. In one experiment, [¹⁴C]octylglucoside and ³H-labeled virus proteins were used to check the removal of the detergent. It was found that 95% of the octylglucoside was dialyzed out during the first 10 h, and that <0.06% of the ¹⁴C-activity was left in the dialysis bag after 36 h. The remaining radioactivity corresponded to <0.05 mg octylglucoside/mg protein.

Sucrose Gradient Centrifugation

The dialyzed material was analyzed by centrifugation in a 5–20% sucrose gradient with a 60% sucrose cushion at the bottom (Fig. 4). The lipid and the protein sedimented as a broad heterogeneous zone. Lipid dialyzed in the absence of protein barely entered the gradient under the same conditions. Little radioactivity was found on top of the 60% sucrose cushion indicating that the material was not in the form of large aggregates. After prolonged centrifugation of the reconstituate in a 15–60% sucrose gradient, 80% of the phospholipid and the protein occurred together in two bands at densities 1.19–1.21 and 1.05–1.08 g/cm³ (Fig. 5). Vesicles containing lipid alone were again recovered in the three top

fractions only. The hemagglutinating activity (a property of the E1 polypeptide, [14]) followed the protein profile (Fig. 5). For further analysis the fractions were pooled so that pools I and III (Fig. 5) contained the two bands, and pool II the intermediate fractions. The buoyant densities and the distribution of lipid and protein in the three pools are shown in Table I. Pool I had a higher protein-to-lipid ratio than the SFV-membrane, whereas in pool III the ratio was lower. When the protein-to-phospholipid ratio in the reconstitution mixture was increased, the protein-to-lipid ratio in pool III increased, whereas it remained constant in pools I and II. Electrophoretic analysis showed that all three pools contained E1, E2 (Fig. 2*b*), and E3 (E3 is poorly labeled with [³⁵S]methionine and does not show unless the other bands are heavily overexposed). It was also found that pool III contained somewhat less and pool I somewhat more E2 polypeptide than whole virus (Fig. 2*b*).

Electron Microscopy

Thin sections of the whole reconstituted material showed vesicles of different sizes (Fig. 6). The vesicles were single-shelled with a unit membrane thickness of 5–6 nm (insets Fig. 6). The proteins bound to the vesicles could be visualized after negative staining as a coat of spike-like projections (7–8 nm long) covering the surface (Fig. 7). The spikes appeared morphologically

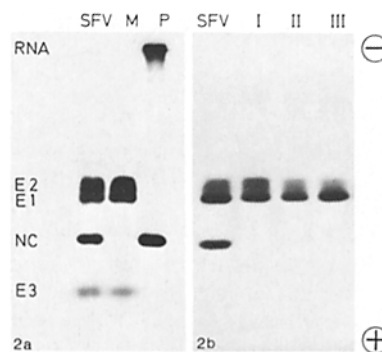


FIGURE 2 Polyacrylamide gel electrophoresis in SDS of the proteins before and after reconstitution. (a) Pooled membrane protein peak, M, and pellet, P, from gradient shown in Fig. 1. SFV, whole virus. E1, E2, and E3 are the polypeptides forming the spike-like projections on the surface of the virus, and NC the protein of the nucleocapsid. (b) Proteins of pools I, II, and III from the isopycnic centrifugation shown in Fig. 5. The bands were visualized by fluorography.

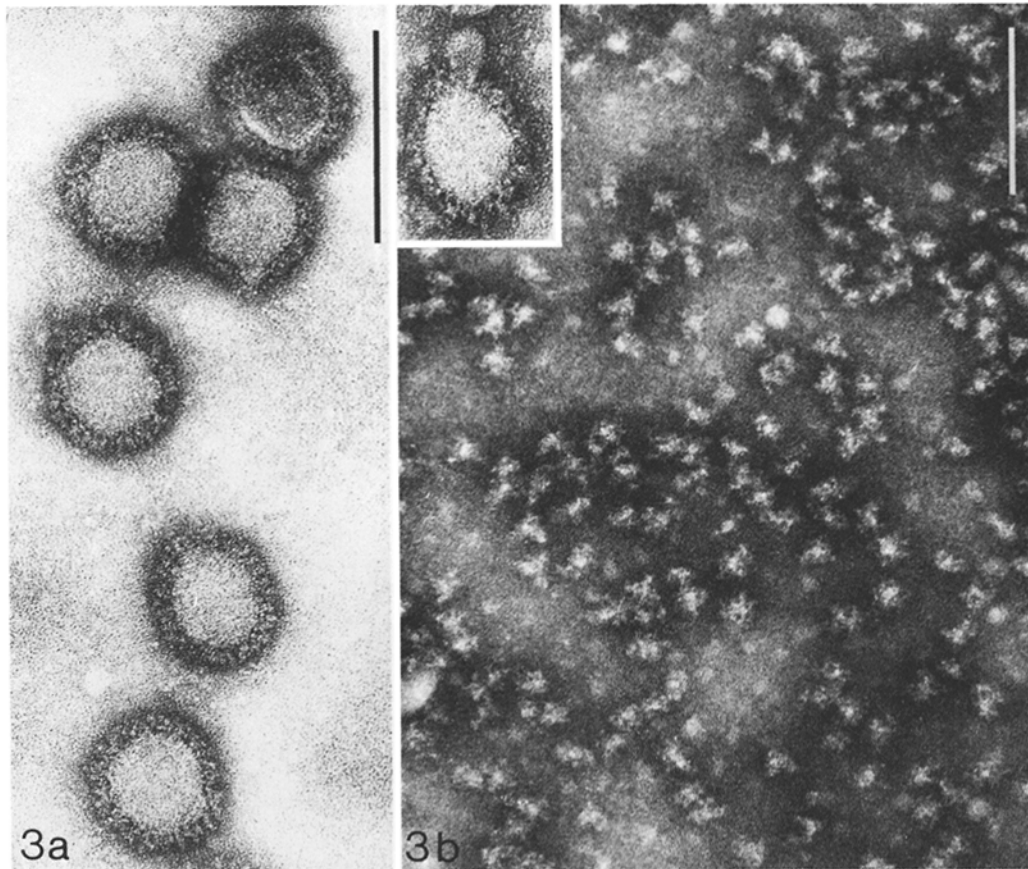


FIGURE 3 (a) SFV as seen after negative staining with 2% phosphotungstic acid at pH 7.0. The virus has a dense outer layer of glycoprotein spikes. At pH below 6.5, the nucleocapsid shrinks, forcing a closer packing of the spikes and the extrusion of a spikeless lipid bleb (inset Fig. 3*b*). (b) Virus spike protein isolated in octylglucoside (Fig. 1). The proteins occur as rosette-like complexes. (a) and inset, $\times 280,000$; (b), $\times 220,000$. Bars, $0.1 \mu\text{m}$.

similar to those on the SFV (Fig. 3*a*). The smallest structures seen were probably too small to be vesicles.

The image areas of the vesicles (excluding the spikes) were measured by planimetry from electron micrographs of negatively stained samples. The areas were mainly within two ranges: $50\text{--}300 \text{ nm}^2$ and $2,500\text{--}6,600 \text{ nm}^2$. The two size ranges are clearly seen in Fig. 8, which shows the number and area distributions within different size classes expressed as mean image radius. If the small vesicles are assumed to be spherical, their radii are $4\text{--}10 \text{ nm}$ (omitting the spikes). If we assume that the large vesicles are flattened out on the grid, we can calculate that the radii of these vesicles in free solution should be $20\text{--}30 \text{ nm}$. The

radius of the viral membrane is 25.8 nm (11). Electron microscopy of negatively stained samples showed that pool I contained the small vesicles (Fig. 9*a*), pool II the intermediate size vesicles (Fig. 9*b*), and pool III mainly the large vesicles (Figs. 10, 11). The average sizes are indicated by arrows in Fig. 8. Because pool I had the lowest, pool II the intermediate, and pool III the highest lipid-to-protein ratio (Table I), we can conclude that increasing vesicle size corresponds to decreasing protein content. When egg lecithin was allowed to reassociate in the absence of spike protein, lipid vesicles of the large size were obtained.

Only in the lipid-rich vesicles of pool III could individual projections be discerned (Fig. 11). They had a club-like appearance with a mean

length of 7.7 nm and a mean thickness of 3.4 nm. Assuming the projections to be cylindrical in shape and having a partial specific volume of 0.72 (31), they could be calculated to have a mol wt of $6 \cdot 10^4$. Other experiments suggested that the individual projections may be E1 polypeptides. When isolated E1 was reconstituted by the octylglucoside dialysis method, the products consisted mainly of lipid-protein vesicles of large size (Fig. 12a). The spikes were well resolved and had a club-like appearance similar to that of the spikes in pool III (Figs. 10, 11). That the individual spikes in the pool III vesicles may represent E1 is also supported by the fact that this polypeptide is enriched in pool III (Fig. 2b). Attempts to reconstitute isolated E2 polypeptides were unsuccessful, as this protein tended to form oligo-

meric complexes which did not combine with lipids to form vesicles.

Large, multishelled structures were occasionally seen in thin sections and negatively stained specimens (Fig. 12b, c). They had unit membranes (mean thickness ~ 5.4 nm) with spikes projecting from both faces. The repeat distance was 18–20 nm, and the spike length and thickness 7 and 3 nm, respectively.

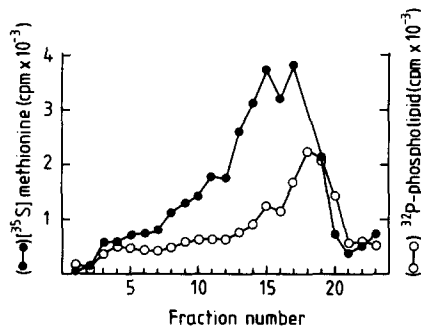


FIGURE 4 Velocity centrifugation of the reconstitute. The sample (100 μ l) was layered onto a 13-ml 5–20% sucrose gradient containing 0.05 M Tris-HCl pH 7.4 and 0.1 M NaCl. To arrest large and aggregated particles, there was a 1-ml 60% sucrose cushion at the bottom. The gradient was centrifuged in an International 488-rotor at 4°C for 2.5 h at $190 \cdot 10^3 \times g$. Fractions were collected from the bottom and 15 μ l aliquots assayed for radioactivity. Bottom to the left.

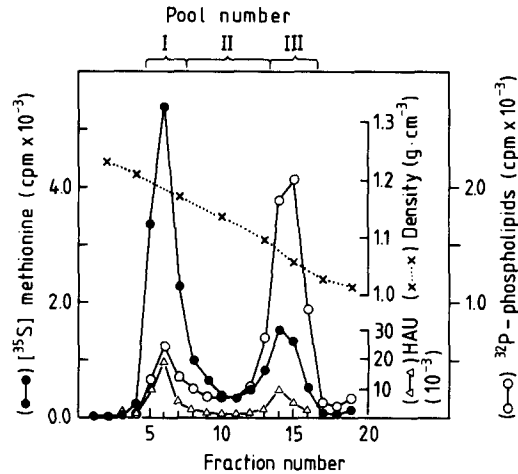


FIGURE 5 Isopycnic centrifugation of the reconstitute. The sample (800 μ l) was layered on a 4-ml 15–60% sucrose gradient containing 0.05 M Tris-HCl pH 7.4 and 0.1 M NaCl. The gradient was centrifuged at 4°C in a SW 50.1 Beckman rotor at $190 \cdot 10^3 \times g$ for 24 h. Fractions were collected from the bottom and aliquots were used for radioactivity measurement (15 μ l), sucrose determination (15 μ l), and hemagglutination assay (10 μ l). Pools I, II, and III were used for further characterization. The specific activities were $2 \cdot 4 \times 10^6$ cpm 35 S-per milligram protein and $1 \cdot 1 \times 10^6$ cpm 32 P-per milligram phospholipid. Bottom to the left.

TABLE I
The Lipid and Protein Compositions of the Reconstituted Membranes*

	Buoyant density	Protein of total	Lipid of total	Phospholipid/protein	Moles phospholipid/ 1 $\cdot 10^6$ g protein
	<i>g/cm³</i>	%	%	<i>mg/mg</i>	
Pool I‡	1.19–1.21	58 \pm 4	11 \pm 3	0.22 \pm 0.03	28 \pm 3
Pool II‡	1.10–1.17	22 \pm 3	16 \pm 5	0.89 \pm 0.04	115 \pm 5
Pool III‡	1.05–1.08	18 \pm 2	61 \pm 2	3.5 \pm 0.7	448 \pm 86
SFV-membrane§	—	—	—	0.6	64

* Averages \pm SE of four separate experiments using 1 mg phospholipid/mg protein in the reconstitution mixture. The compositions were calculated from the specific radioactivities.

‡ The pools were taken from isopycnic sucrose gradients (Fig. 5).

§ From reference 21.

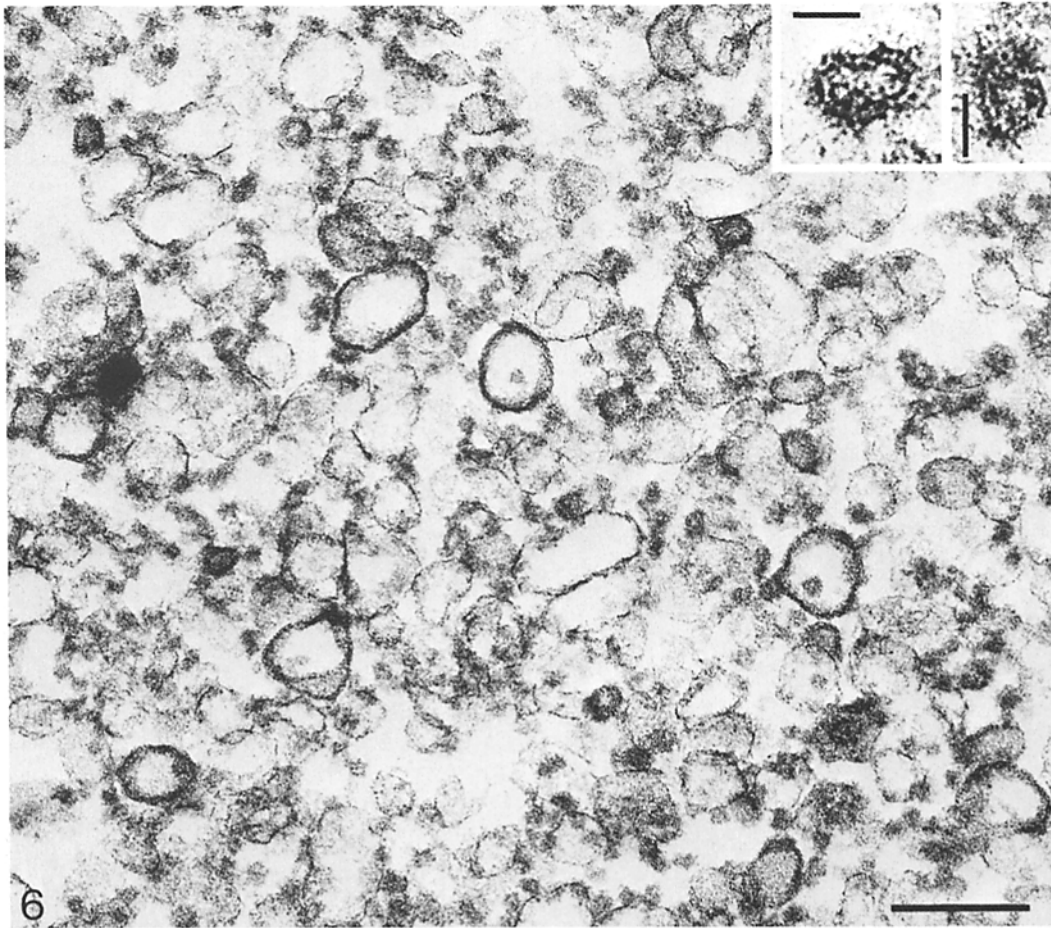


FIGURE 6 Electron microscopy of a thin section of pelleted reconstituted membranes. All vesicles have a single membrane and even the small ones have a unit membrane (see insets). The spike proteins are not resolved with this technique. $\times 180,000$; insets, $\times 440,000$. Bars, $0.1 \mu\text{m}$, and in the insets, 20 nm .

Mode of Attachment and Orientation of the Spike Proteins

When intact SFV is treated with thermolysin, the spikes disappear, but hydrophobic peptides from E1 and E2 with an apparent mol wt of $6 \cdot 10^3$ remain in the membrane (38). These peptides are responsible for anchoring the spikes to the lipid bilayer. To test whether the spikes were similarly attached to the reconstituted vesicles, we treated material from pool I, II, and III with thermolysin. After digestion, the vesicles were isolated from the enzyme and liberated peptides by flotation (Fig. 13). Electron microscopy of the isolated vesicles showed that all the spikes had disappeared from the surface. Analysis of the

protein content by electrophoresis gave two main bands (Fig. 14), one corresponding to E1 and E2 (not resolved in this gel system), and another broad band with the same mobility as the hydrophobic peptides (HF) obtained from SFV after thermolysin treatment. The peptides of the thermolysin-digested vesicles also resembled the hydrophobic virus peptides in being chloroform-methanol soluble.

More than 99% of the E1 and E2 present in pool I were digested by externally added thermolysin. In pool II, 94% was accessible to the enzyme. If the vesicles in these pools are sealed, as is suggested by the thin section electron microscopy (Fig. 6), the spikes must be facing outwards. On the other hand, in pool III the spikes may be

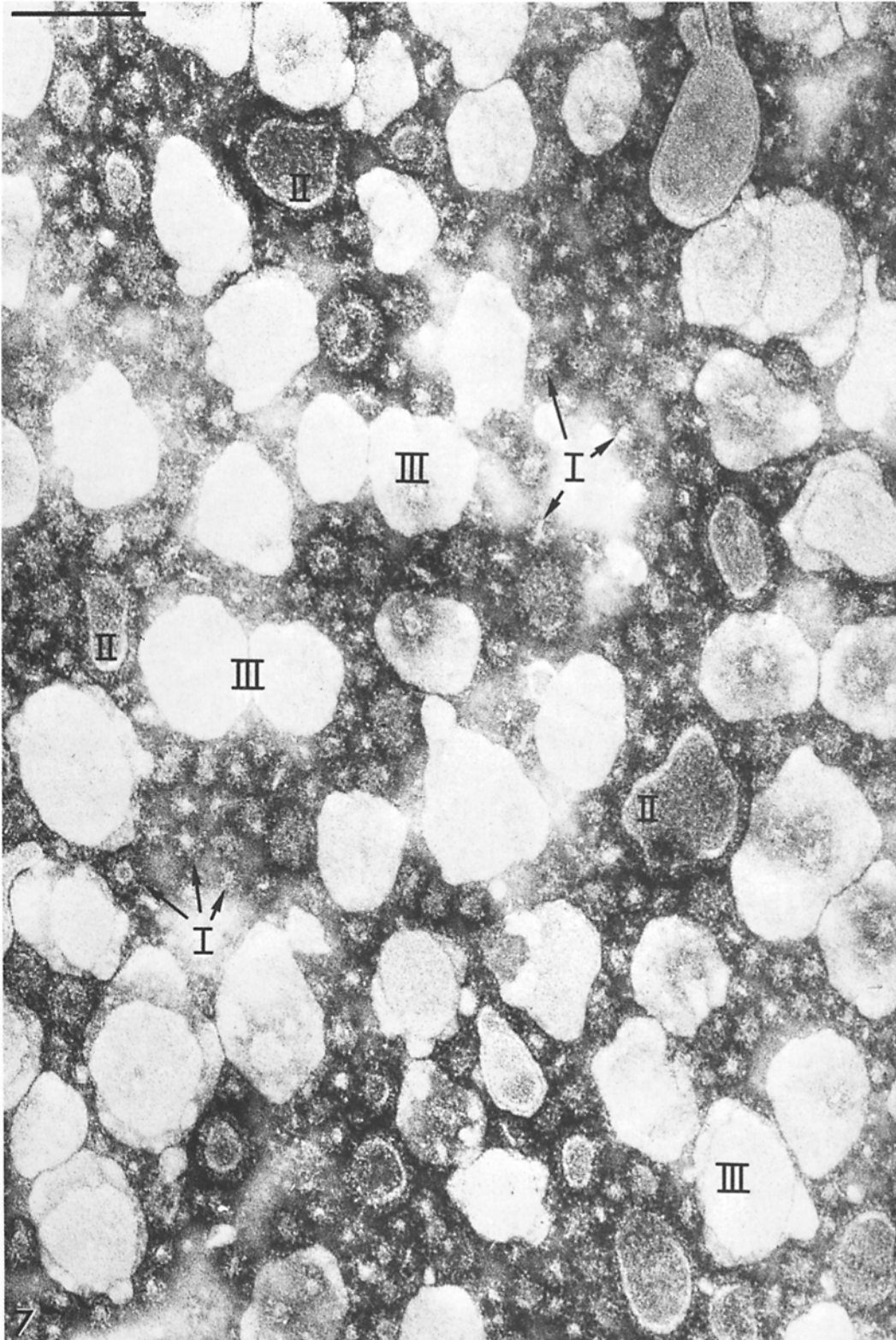


FIGURE 7 Survey picture of the reconstituted membranes as revealed after negative staining. Three main size classes can be seen as indicated by roman numerals (I, II, and III). All membranes have spike-like projections similar to those of the SFV (Fig. 3a). $\times 200,000$. Bar, $0.1 \mu\text{m}$.

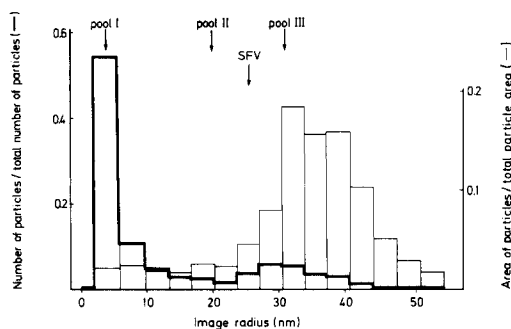


FIGURE 8 Size distribution of the reconstituted membranes as determined by planimetry on electron micrographs after negative staining. The radii given do not include the spikes. The graphs represent data from six electron micrographs comprising 1,600 particles (cf. Fig. 7). The radii (r) were calculated from the measured areas (A) using the relation $A = \pi r^2$. The radius of the virus (from center to the outer bilayer surface, [11]) and the average image radii of the particles in pool I, II and III (Fig. 5) are indicated by arrows.

more symmetrically distributed on both faces of the bilayers, as only 70% of the spike proteins were accessible to the protease.

DISCUSSION

Octylglucoside has several properties which make it suitable for reconstitution experiments. Being nonionic, it is mild in its effects on proteins (3, 37), it poses no restrictions on the choice of buffers, and it does not interfere with the arrangement of charged membrane components during reconstitution. Because of the exceptionally high critical micellar concentration (20–25 mM, cf. ref. 28) it can be rapidly removed by dialysis. It effectively solubilizes membrane lipids and integral membrane proteins (3, 37), and unlike most other nonionic detergents, it is a structurally defined compound.

We found that when SFV membrane proteins were reconstituted with egg lecithin using octylglucoside, two main classes of vesicles were obtained. These could be isolated according to density by isopycnic sucrose gradient centrifugation. The dense vesicles and particles had an average of 28 and the light vesicles 400 phospholipids/spike protein complex (in the viral membrane there are 64 phospholipid cholesterol pairs/spike protein complex (21)). Electron microscopy showed that the dense vesicles were smaller and the light vesicles were equal in size or larger than the SFV.

After digestion with thermolysin, hydrophobic peptides remained in the vesicle membranes suggesting a hydrophobic anchoring of the spike proteins. The accessibility of virtually all proteins in the dense vesicles to thermolysin suggested that the spike proteins were only oriented outwards as in the native virus, whereas the light vesicles appeared to have spike proteins also on the inside of the membrane or in the included space.

The morphology of the spikes on the surface of the SFV (and other toga viruses) is difficult to resolve by electron microscopy (Reference 6, Fig. 3a). However, on the surface of the lipid-rich reconstituted membranes it was possible to see individual club-like projections in addition to more tightly packed spike regions. The size of the single projections corresponded to that of a protein of $\sim 6 \times 10^4$ mol wt. When E1 was reconstituted alone, spikes with the same appearance were seen. It is therefore probable that the projections on the lipid-rich reconstituted membranes represent E1. This is also supported by the fact the lipid-rich vesicles were enriched in E1 polypeptides. Electron microscope studies have suggested that the projections on SFV have a V-shaped structure (6). Recent cross-linking experiments have shown that the spike polypeptides in the SFV membrane occur as complexes of one of E1, E2, and E3 each.¹ What we see as individual spike structures may thus represent one of the arms in the V.

In vivo, the virus acquires its membrane through a budding process at the host cell plasma membrane. In this process the nucleocapsid is enveloped by a modified area of the plasma membrane enriched in spike proteins (1). The nucleocapsid interacts with that part of the spike protein that is localized on the inside of the membrane, and this interaction probably drives the budding process (9). In the finished viral membrane, the number of E1, E2, and E3 complexes equals that of nucleocapsid proteins (9). On the basis of these results, it seems likely that the nucleocapsid not only determines the diameter of the virus but also the lipid-to-protein ratio. When the nucleocapsids are absent, as is the case during reconstitution, the vesicle size and the lipid-to-protein ratio are determined by other factors. The reconstitution yielded vesicles with

¹ Ziemiecki, A., and H. Garoff. Manuscript in preparation.

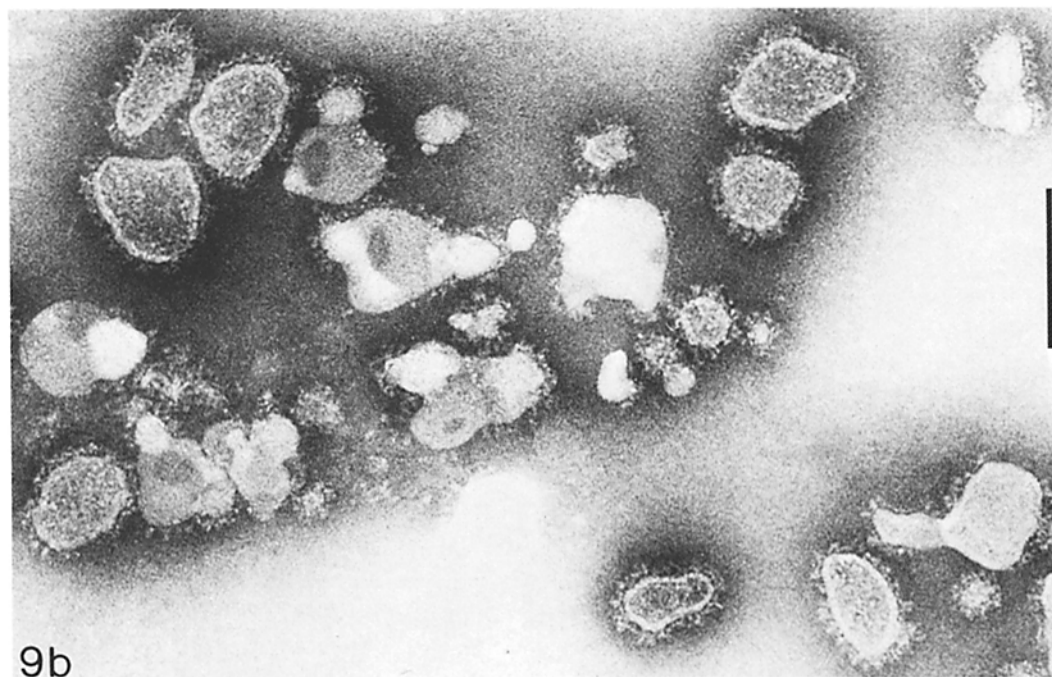
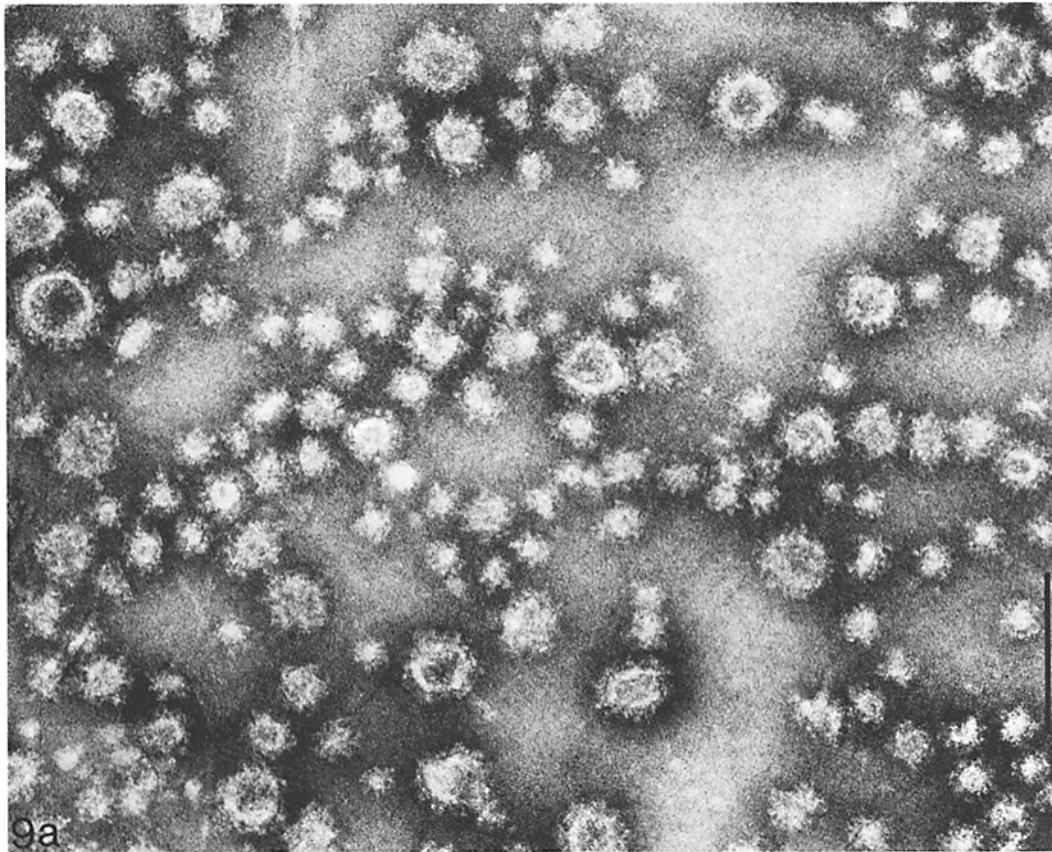


FIGURE 9 Negatively stained fractions of the reconstituted membranes isolated by isopycnic centrifugation (Fig. 5). (a) Pool I, the dense membrane fraction, consisting of small vesicles, and (b) pool II, intermediate density fraction, containing vesicles in the size range of the virus. The spikes around the edges of the vesicles can be easily recognized. $\times 210,000$. Bars, $0.1 \mu\text{m}$.

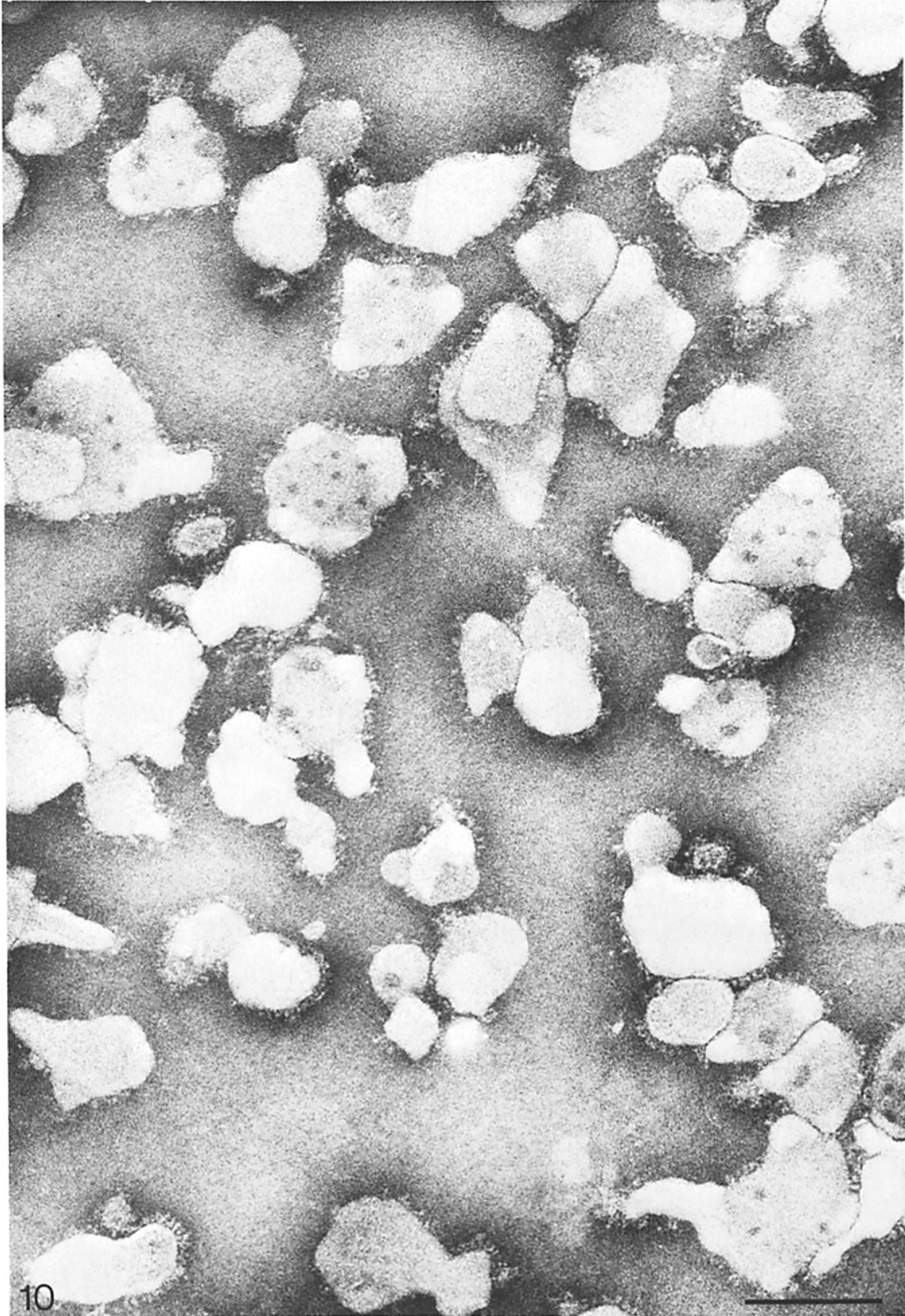


FIGURE 10 Negatively stained fraction (pool III) of the reconstituted membranes isolated by isopycnic centrifugation (Fig. 5). This light membrane fraction consists of large vesicles with a sparse distribution of spikes. $\times 210,000$. Bar, $0.1 \mu\text{m}$.

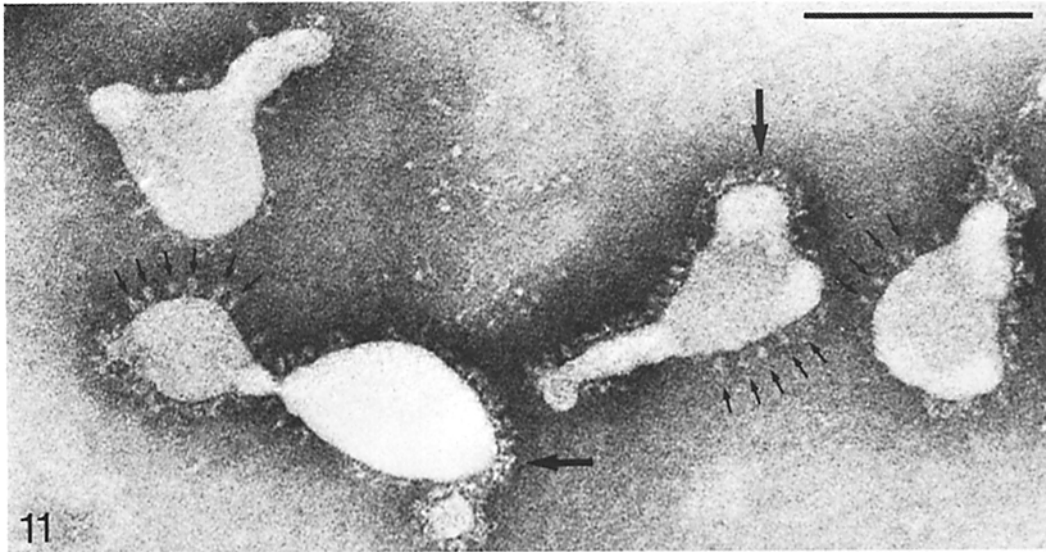


FIGURE 11 Negatively stained vesicles of pool III at high magnification. Some spike proteins are densely clustered giving an appearance similar to that of the surface of the viral membrane (large arrows). Other spike proteins can be seen as irregularly spaced single club-like projections (small arrows). The appearance of these projections are similar to those seen on membranes reconstituted with the polypeptide E1 (Fig. 12a). $\times 300,000$. Bar, $0.1 \mu\text{m}$.

higher as well as lower protein-to-lipid ratio than in the virus membrane. The fact that vesicles with more protein than in the viral membrane could be obtained by the octylglucoside dialysis method (and with the other detergents tested) indicated that the spikes in the viral membrane cannot have maximal packing density. This is further supported by the finding that at low pH the nucleocapsid shrinks (35) and large spike-free lipid blebs are seen protruding from the virus particle resulting in the decrease of viral diameter (Reference 6, Fig. 3b inset).

It may be significant that the majority of reconstituted vesicles were smaller than the virus and smaller than lipid vesicles that had been formed in the absence of protein, and that vesicle size decreased with increasing protein content. Thus, the protein seems to increase membrane curvature. No such effect was observed with the *Bacillus licheniformis* membrane penicillinase which was reconstituted under the same conditions.² This property of the spike proteins may be important in stabilizing the high curvature of the viral membrane. Many of the vesicles with lipid-to-

protein ratios equivalent to that of the viral membrane were in fact approximately of virus size.

It remains to be seen whether the spike proteins in the reconstituted vesicles actually span the bilayer the way they do in the virus, and to what extent the subunit structure of the proteins is intact. It will furthermore be of interest to find out whether the dense and the light vesicles arise by different mechanisms (as is suggested by preliminary results), and to study the effects of different lipid compositions. To characterize the biological properties of the vesicles, we are presently investigating the effects they have on cells, to the surface of which the vesicles can be shown to bind avidly.

The authors wish to thank Karin Goldman, Eva Bolzau, and Signe Mähler for their technical assistance, Hilka Virta for producing the virus, and Doctors Werner W. Franke and Kai Simons for their valuable advice. We are indebted to Dr. Lennart Krabich (Institute for Medical Chemistry, Lund, Sweden) for the synthesis of β -octylglucoside and [^{14}C] β -octylglucoside.

Erik Fries received support from Blanceflor Boncompagni-Ludovisi, born Bildt, Foundation.

Received for publication 23 May 1977, and in revised form 12 August 1977.

² Simons, K., A. Helenius, and M. Sarvas. Manuscript in preparation.

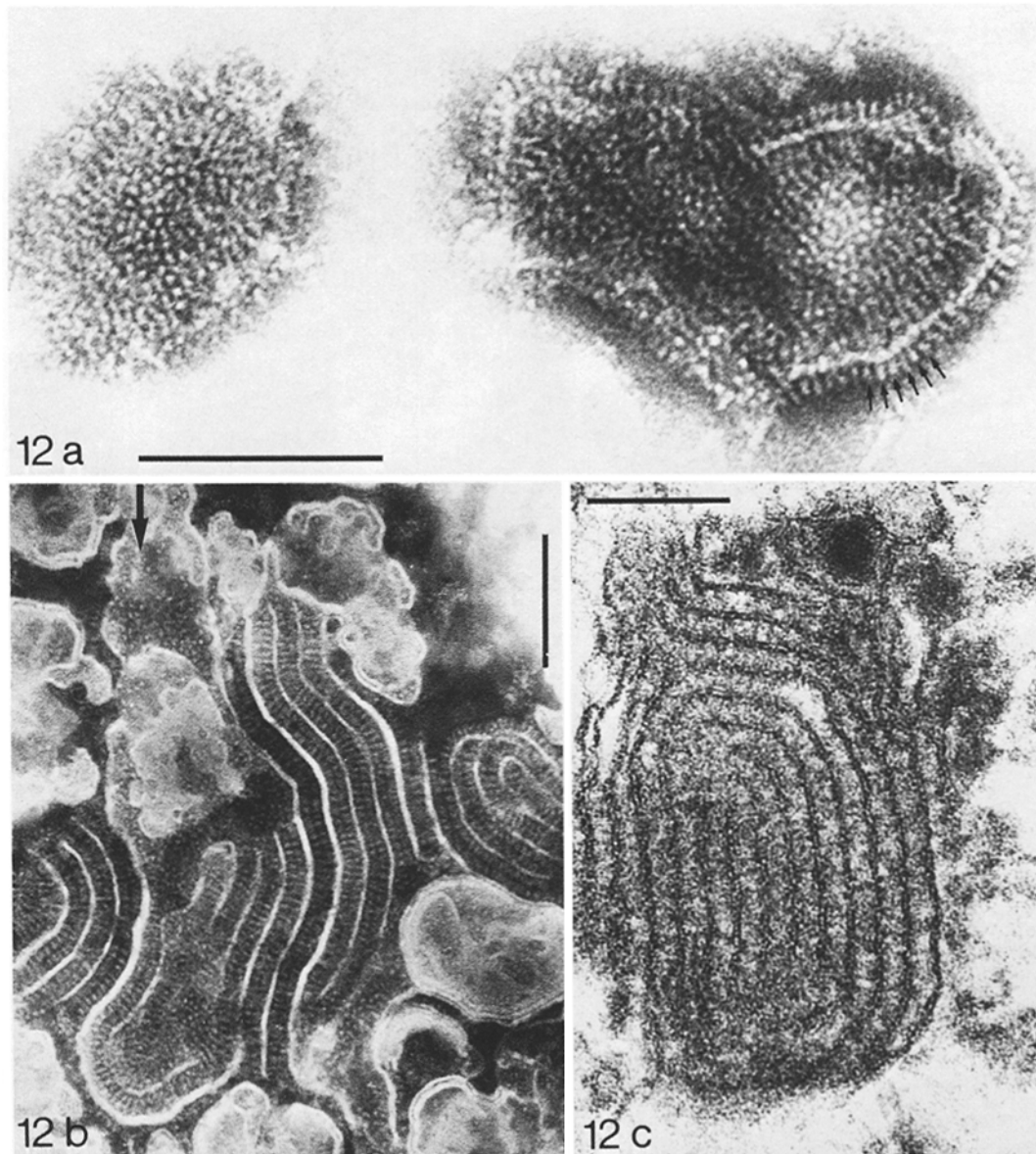


FIGURE 12 (a) Reconstituted membranes obtained with isolated E1 and egg lecithin. At the periphery of the vesicles the proteins are seen in side view as club-like projections. From above, the proteins appear as small white dots with a mean center-to-center distance of 50–58 Å. (b) and (c) Large aggregates of membranes arranged in parallel occasionally found in the total reconstituate. After negative staining, (b) single spikes can be seen as regularly spaced club-like projections or as small white spots. After thin-sectioning, (c) these aggregates show a unit membrane character. (a) $\times 330,000$, (b) and (c) $\times 190,000$. Bars, $0.1 \mu\text{m}$.

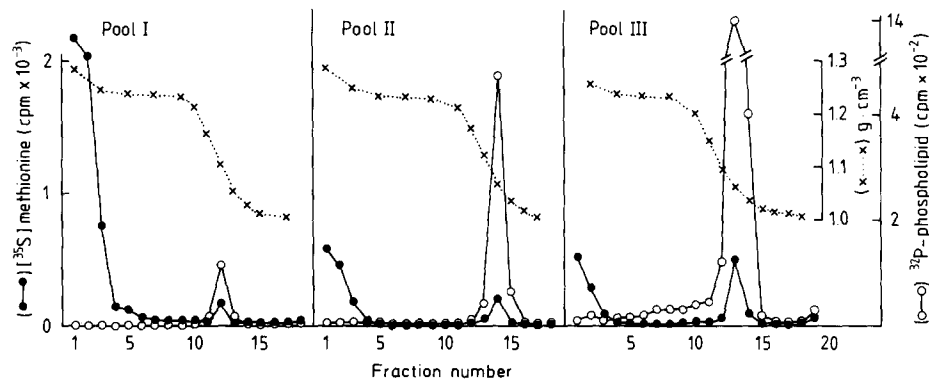


FIGURE 13 Isolation of the reconstituted membranes by flotation after digestion with thermolysin. The digest with added sucrose and EDTA was placed at the bottom of the centrifuge tube and overlaid with sucrose and buffer. Centrifugation was performed in a Beckman SW 50.1 rotor at 4°C for 10 h at $190 \cdot 10^8 \times g$. Fractions were collected from below, and aliquots (25 μ l) were used for radioactivity measurement. Bottom to the left.

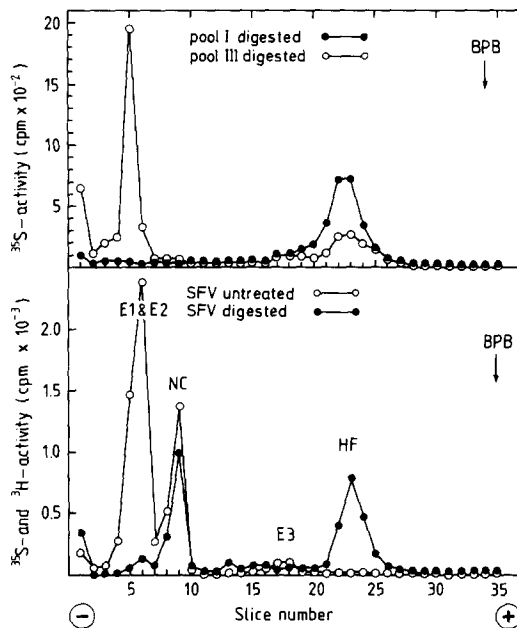


FIGURE 14 Comparison of polypeptides remaining after thermolysin treatment in reconstituted membranes and in SFV using polyacrylamide gel electrophoresis in SDS. (a) Thermolysin digested pool I and III (Fig. 13), (b) thermolysin digested [3 H]isoleucine, leucine, valine SFV, and untreated [35 S]methionine SFV. HF, hydrophobic peptide fragments obtained from the virus membrane after digestion (38); NC, nucleocapsid protein. The gel slices were solubilized and measured for radioactivity as described (38).

REFERENCES

- ACHESON, N. H., and I. TAMM. 1967. Replication of Semliki Forest virus: an electron microscopic study. *Virology*. **32**:128-143.
- ALMEIDA, J. D., C. M. BRAND, D. C. EDWARDS, and T. D. HEATH. 1975. Formation of virosomes from influenza subunits and liposomes. *Lancet*. **2**:899-901.
- BARON, C., and T. E. THOMPSON. 1975. Solubilization of bacterial membrane proteins using alkylglucosides and dioctanoyl phosphatidylcholine. *Biochim. Biophys. Acta*. **382**:276-285.
- BARTLETT, G. R. 1959. Colorimetric assay for free and phosphorylated glyceric acids. *J. Biol. Chem.* **234**:469-471.
- BECKER, R., A. HELENIUS, and K. SIMONS. 1975. Solubilization of Semliki Forest virus membrane with sodium dodecyl sulfate. *Biochemistry*. **14**:1835-1841.
- v. BONSDORFF, C.-H. 1973. The structure of Semliki Forest virus. *Commentat. Biol. Soc. Sci. Fenn.* **74**:1-53.
- CLARKE, D. H., and J. CASALS. 1958. Techniques for hemagglutination with arthropod-borne viruses. *Am. J. Trop. Med. Hyg.* **7**:561-573.
- FOLCH, J., M. LEES, and G. H. SLOANE STANLEY. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**:497-509.
- GAROFF, H., and K. SIMONS. 1974. Location of spike glycoproteins in the Semliki Forest virus membrane. *Proc. Natl. Acad. Sci. U. S. A.* **71**:3988-3992.
- GAROFF, H., K. SIMONS, and O. RENKONEN. 1974. Isolation and characterization of the membrane proteins of Semliki Forest virus. *Virology*. **61**:493-504.
- HARRISON, S. C., A. JACK, D. GOODENOUGH, and B. M. SEFTON. 1974. Structural studies of spherical viruses. *J. Supramol. Struct.* **2**:486-495.
- HELENIUS, A., and C.-H. v. BONSDORFF. 1976. Semliki Forest virus membrane proteins. Prepara-

- tion and characterization of spike complexes soluble in detergent-free medium. *Biochim. Biophys. Acta.* **436**:895-899.
13. HELENIUS, A., and H. SÖDERLUND. 1973. Stepwise dissociation of the Semliki Forest virus membrane with Triton X-100. *Biochim. Biophys. Acta.* **307**:287-300.
 14. HELENIUS, A., E. FRIES, H. GAROFF, and K. SIMONS. 1976. Solubilization of the Semliki Forest virus membrane with sodium deoxycholate. *Biochim. Biophys. Acta.* **436**:319-334.
 15. HOSAKA, Y. 1977. Reconstitution of virus membranes. In *Cell Membranes and Viral Envelopes*. H. A. Blough and J. M. Tiffany, editors. Academic Press, Inc. New York. In press.
 16. HOSAKA, Y., and Y. K. SHIMIZU. 1972. Artificial assembly of envelope proteins of HVJ (Sendai virus). I. Assembly of hemolytic and fusion function from envelopes solubilized by Nonidet P40. *Virology.* **49**:640-646.
 17. KÄÄRIÄINEN, L., K. SIMONS, and C.-H. v. BONS-DORFF. 1969. Studies on subviral components of Semliki Forest virus. *Ann. Med. Exp. Biol. Fenn.* **47**:235-248.
 18. KAGAWA, Y., and E. RACKER. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. XXV. Reconstitution of vesicles catalyzing [³²P]adenosine triphosphate exchange. *J. Biol. Chem.* **246**:5477-5487.
 19. KASAHARA, M., and P. C. HINKLE. 1976. Reconstitution of D-glucose transport catalyzed by a protein-fraction from human erythrocytes in sonicated liposomes. *Proc. Natl. Acad. Sci. U. S. A.* **73**(Suppl. 2):376-396.
 20. KRAHL, M. E., and C. F. CORI. 1954. *Biochem. Prep.* **3**:33.
 21. LAINE, R., H. SÖDERLUND, and O. RENKONEN. 1973. Chemical composition of Semliki Forest virus. *Int. Virol.* **1**:110-118.
 22. LASKEY, R. A., and A. D. MILLS. 1975. Quantitative film detection of [³H] and [¹⁴C] in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**:335-341.
 23. MEISSNER, G., and S. FLEISCHER. 1974. Dissociation and reconstitution of functional sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **249**:302-309.
 24. NEVILLE, D. M. 1971. Molecular weight determination of protein-dodecylsulphate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* **246**:6328-6334.
 25. NOLLER, C. R., and W. C. ROCKWELL. 1938. The preparation of some higher alkylglucosides. *J. Am. Chem. Soc.* **60**:2076-2077.
 26. PFEFFERKORN, E. R., and D. SHAPIRO. 1974. Reproduction of Togaviruses. In *Comprehensive Virology*. H. Fraenkel-Conrat and R. Wagner, editors. Plenum Publishing Corporation, New York. **2**:171-220.
 27. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electronopaque stain in electron microscopy. *J. Cell Biol.* **17**:208-212.
 28. SHINODA, K., T. YAMAGUCHI, and R. HORI. 1961. The surface tension and critical micellar concentration in aqueous solution of β -D-alkyl glucosides and their mixtures. *Bull. Chem. Soc. Jpn.* **34**:237-241.
 29. SIMONS, K., and L. KÄÄRIÄINEN. 1970. Characterization of the Semliki Forest virus core and envelope protein. *Biochem. Biophys. Res. Commun.* **38**:981-988.
 30. SIMONS, K., H. GAROFF, and A. HELENIUS. 1977. The glycoproteins of the Semliki Forest virus membrane. In *Membrane Proteins and their Interaction with Lipids*. R. A. Capaldi, editor. Marcel Dekker, Inc. New York. 207-234.
 31. SIMONS, K., A. HELENIUS, and H. GAROFF. 1973. Solubilization of the membrane proteins from Semliki Forest virus with Triton X-100. *J. Mol. Biol.* **80**:119-133.
 32. SIMONS, K., H. GAROFF, A. HELENIUS, L. KÄÄRIÄINEN, and O. RENKONEN. 1974. Structure and assembly of virus membranes. In *Perspectives in Membrane Virology*. S. Estrada and C. Gitler, editors. Academic Press, Inc. New York. 45-70.
 33. SIMONS, K., L. KÄÄRIÄINEN, O. RENKONEN, C. G. GAHMBERG, H. GAROFF, A. HELENIUS, S. KERÄNEN, R. LAINE, M. RANKI, H. SÖDERLUND, and G. UTERMANN. 1976. Semliki Forest virus envelope as a simple membrane model. In *Membrane Mediated Information*. P. W. Kent, editor. Medical and Technical Publishing Company Ltd., Lancaster, **2**:81-99.
 34. SIMMONS, D. T., and J. H. STRAUSS. 1972. Replication of Sindbis virus. I. Relative size and genetic content of 26S and 49S RNA. *J. Mol. Biol.* **71**:599-613.
 35. SÖDERLUND, H., L. KÄÄRIÄINEN, C.-H. v. BONS-DORFF, and P. WECKSTRÖM. 1972. Properties of Semliki Forest virus nucleocapsids. II. An irreversible contraction by acid pH. *Virology.* **47**:753-760.
 36. STRITTMATTER, P., and M. J. ROGERS. 1975. Apparent dependence of interactions between cytochrome b₅ and cytochrome b₅ reductase upon translational diffusion in dimyristoyl-lecithin liposomes. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2658-2661.
 37. STUBBS, G. W., H. G. SMITH, and B. J. LITMAN. 1976. Alkyl glucosides as effective solubilizing agents for bovine rhodopsin. A comparison with several commonly used detergents. *Biochim. Biophys. Acta.* **426**:46-56.
 38. UTERMANN, G., and K. SIMONS. 1974. Studies on the amphipathic nature of the membrane proteins in Semliki Forest virus. *J. Mol. Biol.* **85**:569-587.
 39. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407-408.