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NOTES

Incorporation of Influenza Virus M-Protein into Liposomes

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M-protein from influenza virus vaccine was purified by sodium dodecyl sulfate-gel chromatography and incorporated into liposomes by solubilization with octylglucoside and subsequent dialysis. Liposomes containing M-protein formed a distinct population with a density of 1.22 g/ml on sucrose-gradient centrifugation, regardless of the net charge on the liposomes. Treatment of the liposomes by freeze-fracture followed by electron microscopic examination showed multilamellar structures in those liposomes without M-protein; liposomes containing M-protein were mulberry-like structures which appeared unilamellar. These studies show incorporation of M-protein into the lipid bilayer.

The M (matrix or membrane)-protein is a major protein constituent of three groups of enveloped viruses: orthomyxoviruses (influenza viruses), paramyxoviruses, and rhabdoviruses. These viruses are defined as enveloped because the external glycoprotein antigens are situated in a lipid bilayer. In the case of influenza virus, M-protein has been reported to form an electron-dense layer inside the lipid bilayer (2, 8, 9). Although the M-protein is the most abundant protein in the virus particle, relatively little is known about its function, perhaps in part due to its hydrophobic properties (11) and the difficulties in applying classical biochemical techniques to studies of such a protein.

Sodium dodecyl sulfate (SDS)-gel chromatography permits the isolation of milligram quantities of M-protein and other major viral proteins (5, 7). Since the influenza virion is an enveloped virus, we examined the interaction of M-protein with artificial lipid bilayers, or liposomes. An artificial system of M-protein and lipid should provide an interesting model for studying the function of M-protein in the virion and its possible interaction with the glycoprotein spikes or the ribonucleoprotein of influenza virus or both.

M-protein was purified from a commercial lot of X-38 (Heq1N2) virus which had been Formalin treated for vaccine use (7). The vaccine was the gift of Lederle Laboratories, Pearl River, N.Y. M-protein was isolated by SDS-gel chromatography on Bio-Gel A-5m (Bio-Rad Laboratories) columns after disruption of the virus preparation with 10% SDS. As previously described (7), M-protein eluted from the A-5m column under nonreducing conditions with a molecular weight of 25,000. All of the major proteins of influenza virus, including NP, HA1, HA2, and M, could be purified from this preparation in milligram quantities; modification due to formaldehyde exposure appeared minimal (7). SDS-polyacrylamide gel electrophoresis of purified M-protein is shown in Fig. 1 (6). No carbohydrate could be detected as determined by periodic acid-Schiff reagent (6).

SDS was removed by continuous flushing of the M-protein preparation while the protein was maintained in a large volume to promote formation of the monomer form of SDS (16). An Amicon model 202 ultrafiltration unit equipped with a PM-10 membrane was connected to an RG12 reservoir (Amicon Corp.), and 4 liters of distilled water was flushed through the M-protein solution. The M-protein sample was shown to be free of SDS by the absence of hemolysis of an equivalent volume of a 0.5% solution of erythrocytes in microtiter plates; this indicates a concentration below 0.00045% SDS, the lower limit of detection in this test.

The appropriate phospholipid mixtures were mixed with M-protein and solubilized by the use of octyl glucoside (Sigma Chemical Co.) in a protein/total lipid/octyl glucoside ratio of 1:5:25 (3, 12). All lipids employed were products of GIBCO Laboratories. Liposomes were prepared with ovolecithin alone or with a mixture of lipids to provide a net negative or a net positive charge. The liposomes containing a net negative charge

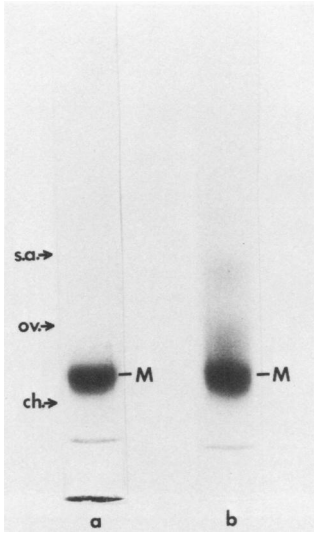


FIG. 1. *SDS-polyacrylamide gel electrophoresis (nonreducing conditions) of M-protein preparation (a) and M-protein liposomes (b) isolated from the sucrose density gradient with a density of 1.22 g/ml (see Fig. 2). Approximately 10 μg of protein was applied to each gel. Positions of molecular weight markers are noted on (a): serum albumin (s.a.), 68,000; ovalbumin (ov.), 43,000; and chymotrypsinogen (ch.), 25,700 (19). M-protein has an apparent molecular weight of 29,000.*

were prepared in a lecithin/dicetyl phosphate/cholesterol molar ratio of 9:2:1; the liposomes containing a net positive charge were prepared in the same molar ratio with stearylamine substituted for dicetyl phosphate. Liposomes formed on dialysis of octyl glucoside from the preparation versus 0.002 M $CaCl_2$ at 4°C for 48 h; dialysis medium was replaced, and dialysis was continued for an additional 2 days.

After formation of the liposomes by dialysis, the liposomes were fractionated by centrifugation or flotation on sucrose gradients. The liposome mixture was either overlaid in 1 ml on a 10 to 30% sucrose gradient with a 1.5-ml sucrose cushion of 60% sucrose or placed in a layer of 40% sucrose between the gradient and the 60% sucrose cushion, and the liposomes were separated by flotation. Centrifugation was performed in an SW 27.1 rotor at 120,000 × *g* for 18 h. Fractions were collected from the gradients and assayed for protein by the Lowry assay (14); 1 drop of SDS was added to each assay to facilitate solubilization of the liposomal preparation. Density of fractions was determined by refractive index measurements. The liposomal preparation was dialyzed versus distilled water to remove sucrose.

M-protein readily associated with lipid vesicles, regardless of the net charge, to form M-protein-containing liposomes. Liposomes with no added protein had a density of 1.03 g/ml (Fig. 2). Heavy-density liposomes (1.20 to 1.22 g/ml) formed in the presence of M-protein for all mixtures of lipids employed, net negative charge, net positive charge, or neutral charge (lecithin only). A light-density band (1.04 g/ml) also formed in the presence of M-protein. M-protein was shown to be present in the heavy-density liposomes (1.22 g/ml) as assayed by SDS-polyacrylamide gel electrophoresis (Fig. 1b); M-protein could not be detected in the light-density band (1.04 g/ml).

Liposomes formed in the presence of M-protein were composed of two discrete populations as examined by sucrose gradient centrifugation, those with a heavy density (1.22 g/ml) which contained M-protein and those with a light density (1.04 g/ml) which contained no detectable M-protein (Fig. 1 and 2). M-protein liposomes did not form a heterogeneous mixture of liposomes of various densities or a single population of uniform density. The presence of two distinct liposomal populations, those containing M-protein (1.22 g/ml) and those lacking M-protein (1.04 g/ml), suggests that a cooperative process is occurring. When some M-protein is incorporated into the liposome, more M-protein partitions into the liposomes to form heavy-density liposomes, leaving a second population of liposomes of light density containing little or no M-protein.

The total M-protein recovered from the heavy-density band after sucrose gradient centrifugation ranged from 35 to 65% of the M-protein added at the start of dialysis. Although lipid content was not measured directly, an approximation of the lipid/protein ratio in the

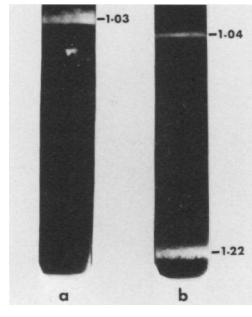
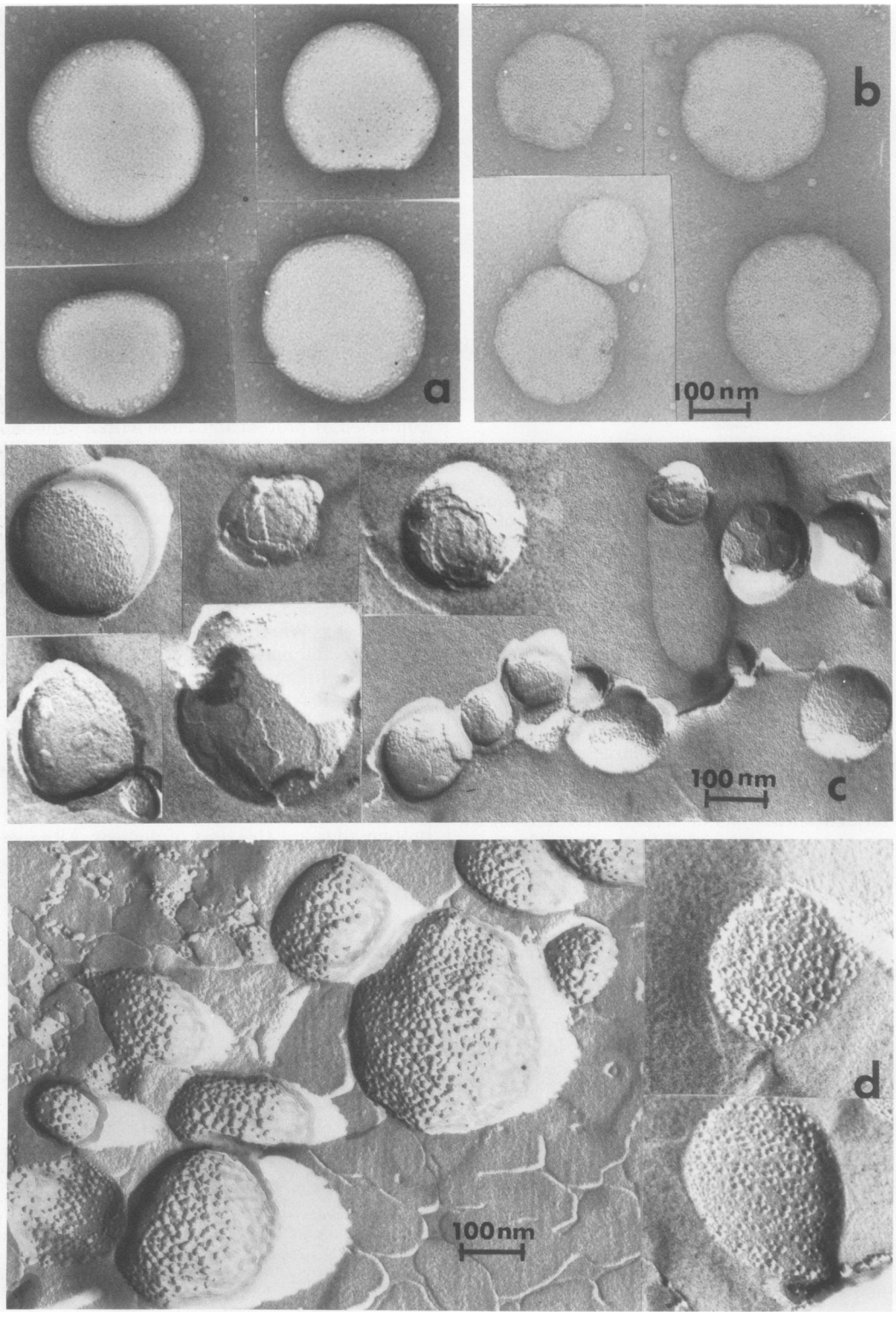


FIG. 2. *Sucrose gradient centrifugation of (a) liposomes without M-protein added and (b) liposomes formed in the presence of M-protein. Note heavy band with a density of 1.22 g/ml in the presence of M-protein on (b). Light-density bands had densities of 1.03 g/ml on (a) and 1.04 g/ml on (b).*


a
b
100 nm
100 nm
c
100 nm
d

heavy-density band could be made as follows. Since M-protein is non-glycosylated, a partial specific volume ($\bar{v}$) of 0.70 cm^3/g can be assumed (15). The contribution of M-protein to increase the density of the liposomes from 1.03 to 1.22 g/ml is equivalent to 1.43 g/ml ($1/\bar{v}$). The density of liposomes in the absence of M-protein is 1.03 g/ml. The total density of the M-protein liposomes (1.22 g/ml) could be described as $d_l(a) + d_p(100 - a) = 1.22$ g/ml; where d_l is 1.03 g/ml or the density of the lipid without M-protein, d_p is 1.43 g/ml of M-protein, a is the percent lipid, and $100 - a$ is the percent protein. This equation gives an estimate of 53% lipid and 47% M-protein contained in the heavy-density liposomes to result in a density of 1.22 g/ml. Vesicles formed from fragmented sarcoplasmic reticulum have been found to consist of 53 mg of lipid per 100 mg of protein, or 35% lipid and 65% protein (4).

M-protein liposomes with a net negative charge appeared to be more stable than positively charged or neutral liposomes, and these were chosen for additional study. After storage at 4°C for 1 week, liposomes with a net positive charge or neutral charge showed considerable loss of the discrete heavy fraction on sucrose gradient centrifugation with formation of heterogeneous lighter components. The M-liposomes with a net negative charge maintained the same distribution on sucrose gradient centrifugation even after 1 week of storage at 4°C.

M-protein liposomes (heavy density, 1.22 g/ml) and liposomes to which no M-protein was added (light density, 1.03 g/ml) were purified on sucrose gradients and examined by electron microscopy after negative staining with uranyl acetate. No obvious differences could be detected between the two preparations (Fig. 3). However, specimens prepared by freeze-fracture for electron microscopy showed dramatic differences in the liposomes with and without M-protein. Liposomes with no M-protein were frequently multilamellar with no specific surface features. Liposomes containing M-protein were distinct mulberry-like structures which appeared to be unilamellar (Fig. 3).

The M-liposomes were studded with 10-nm particles; allowing 1 to 2 nm for shadowing (4), the actual particle size was 8 to 9 nm. Chymotrypsinogen has nearly the same molecular weight as M-protein (25,700) and had an ellipsoidal shape with axes of 5 by 4 by 4 nm as determined by X-ray diffraction (10). This size is considerably smaller than the 8- to 9-nm particles seen in M-protein liposomes and suggests that each particle contains two or more M-protein molecules.

One field (area, 0.1 μm^2) was sampled from the center of each of 13 M-protein-containing liposomes, and the number of "bumps" was counted to determine the particle density of these liposomes. The median density was 3,000/μm^2 with a mean particle density of 2,885/μm^2. The range in particle density was 1,600 to 3,600/μm^2.

The size (8 to 9 nm) and distribution (mean density, 2,885/μm^2) of the particles seen in M-liposomes are similar to those found for intramembranous particles of other biological membranes examined by electron microscopy after freeze-fracture preparation. Segrest and associates (17) found 8-nm particles at a density of up to 4,000/μm^2 in preparations of the membrane-penetrating peptide of the MN glycoprotein in phospholipid vesicles. Freeze-etch preparations of erythrocyte membranes show 7.5-nm particles at a concentration of 4,200 to 4,900 particles per μm^2 (18). Vesicles of fragmented sarcoplasmic reticulum prepared by freeze-fracture show 8-nm intramembranous particles at a density of 3,800 to 3,900/μm^2 (4).

We have demonstrated the incorporation of purified M-protein from an enveloped virus into an artificial lipid bilayer. Glycoprotein "spikes" of influenza virus have been previously shown to insert into artificial membranes (1, 13). Coformation of M-protein liposomes with glycoprotein spikes will provide a useful model system for the study of the assembly of enveloped viruses.

FIG. 3. *Electron micrographs of sucrose gradient-purified liposomes. Upper panels show negative-stained preparations of (a) liposomes without M-protein (d, 1.03 g/ml) and (b) liposomes formed in the presence of M-protein (d, 1.22 g/ml). Staining was performed by placing the liposomal suspension on Formvar carbon-coated grids and treating with a 1% aqueous solution of uranyl acetate. (c) and (d) show specimens of liposomes prepared by freeze-fracture. Liposomes formed in the absence of M-protein (d, 1.03 g/ml) are shown in (c). Liposomes formed in the presence of M-protein (d, 1.22 g/ml) are shown in (d). Freeze-fracture was performed by placing 1 μl of liposomal solution on 3-mm Balzers gold disks in Freon-22 and cooling to −150°C in liquid nitrogen. Cryoprotective agents were not used. Disks were mounted on the specimen stage of a Balzers BAF-301 unit, and the fracture procedure was carried out at −130°C at a pressure of 10^{-6} torr. The fractured surfaces were shadowed with platinum-carbon. The replicas were cleaned with sodium hypochlorite for at least 6 h, rinsed twice in distilled water, and then mounted on electron microscope grids. Specimens were examined in a JEM-100B electron microscope at an instrument magnification of 30,000. Final magnification is 90,000.*

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