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Interaction of Influenza M Protein with Viral Lipid and Phosphatidylcholine Vesicles

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The M protein of influenza is the predominant structural component of the virus. The interactions of this protein with the viral lipid or with other proteins are not known. The ability of M to interact with viral or other lipids was investigated. Purified M was mixed with viral lipid or egg phosphatidylcholine and was incorporated into vesicles (i) by addition of sodium deoxycholate followed by dialysis or (ii) by sonication. Between 90 and 100% of the M became firmly associated with the lipid by either of these two methods, whereas nucleoprotein failed to associate with the vesicles. Firm association also occurred if M was mixed with performed vesicles. Most of the M attached to the vesicles could be hydrolyzed with proteolytic enzymes such as trypsin or thermolysin, except for a small fragment of about 5,000 daltons which remained associated with the lipid vesicles. The ability of fragments of M to interact with lipids was also investigated. Of 13 fragments produced by cleavage with cyanogen bromide, 3 specifically associated with lipid vesicles. The data indicate that a specific portion of the M molecule has a high affinity for lipid bilayers of various origins.

The influenza virus membrane contains two types of surface projections, hemagglutinin and neuraminidase. Hemagglutinin has been shown to be stably attached to the lipid bilayer of the virus by a sequence of hydrophobic amino acids at the C terminus which is inserted into the lipid (8, 36). The neuraminidase appears to be attached in a similar way (21, 40), although sequence data are not yet available.

The membrane or matrix (M) protein of the virus is localized directly beneath the lipid bilayer and appears as a tightly adherent sac surrounding the nucleoprotein (2, 3, 35). Despite the fact that this protein is the major viral component and constitutes approximately 40% of the total protein (9, 34), its structural relationship to the lipid and other viral proteins is unclear. In some preparations, a slight space is observed between M and the lipid bilayer, suggesting that M is not inserted into the lipid (25). Other data obtained by fluorescent dyes embedded into the lipid suggest that M may penetrate into the bilayer (22). The amino acid composition of M (11, 20) and of isolated fragments (32) has been reported, and the sequence of the nucleic acid coding for the protein has now been completed (5, 39a). M has certain characteristics of membrane proteins, such as insolubility in buffers lacking detergents; it is soluble in organic solvents such as acidic chloroform-methanol (11).

The work reported here was carried out to determine whether the M protein of influenza can interact with viral or other lipids. M had a high affinity for lipid since it easily incorporated into lipid vesicles in vitro and attached to these by a segment of about 5,000 daltons. Of 13 fragments produced when M was cleaved at the methionine residues by cyanogen bromide, 3 specifically interacted with lipid vesicles. The data suggest that a specific portion of the molecule has a high affinity for lipid.

MATERIALS AND METHODS

Cells and virus. The WSN strain of influenza virus was grown in chicken embryo fibroblasts and labeled with radioactive amino acids or methionine as previously described (13).

Purification of M and incorporation into vesicles. M was purified by extraction into chloroform-methanol or by mild detergents. Selective extraction into chloroform-methanol was carried out as previously described (11), with one slight modification. The virus suspension was made 0.4 M with respect to sodium chloride before extraction with organic solvent, as this eliminated the trace amounts of nucleoprotein sometimes carried along.

M was purified by mild detergents as follows. WSN virus was disrupted with 1% Nonidet P-40-0.1% deoxycholate-0.02 M NaCl-0.005 M Tris (pH 7.6). The virus was incubated for 3 min at 37°C and put onto a DEAE-cellulose (Brown Co.) column (0.5 by 2.5 cm) equilibrated with 0.07% deoxycholate-1% Nonidet P-

40–0.005 M Tris (pH 7.6). The M protein was excluded from the column. The Nonidet P-40 in the protein solution was exchanged for deoxycholate by binding the protein to a second DEAE-cellulose column equilibrated with buffer containing 0.07% deoxycholate–0.005 M Tris (pH 7.6). The bound material was washed with the same buffer, and M was eluted with buffer containing 1% deoxycholate and 0.5 M NaCl.

Viral lipid was extracted from virus with 20 volumes of chloroform-methanol. Egg phosphatidylcholine (in chloroform) was from P-L Biochemicals. [^{3}H]phosphatidylcholine was from Applied Science Laboratories.

Incorporation of M into vesicles was carried out as follows. Viral lipid or phosphatidylcholine was added to the chloroform-methanol extract of M at a lipid-to-protein ratio of 10:1. Each experiment consisted approximately of 1 to 2 mg of egg phospholipid or 100 μg of viral lipid. The material was dried under a vacuum for 1 h and then suspended in 0.5 to 1.0 ml of buffer (0.1 M NaCl, 0.05 M Tris [pH 7.6], 0.005 M KCl) containing 1% deoxycholate (Calbiochem) and shaken in a Vortex mixer for 5 min. The material was dialyzed for 36 h against several liter changes of 0.1 M NaCl–0.05 M Tris (pH 7.6)–0.005 M KCl–0.002 M NaN_3. Alternatively, M and lipid were dried by vacuum, suspended in buffer without any detergent, mixed 5 min on a Vortex mixer, and sonicated 20 min at 0°C at full power (Raytheon sonic oscillator, model 101).

M which had been isolated from the virus with detergents was added directly to tubes containing viral lipid or phosphatidylcholine which had already been dried by vacuum. The tubes were mixed on a Vortex mixer for 5 min and the deoxycholate was removed by dialysis as outlined above.

Density-gradient centrifugation. Sucrose was added to suspensions of lipid vesicles to make them 45%, and a linear 5 to 30% gradient of sucrose (in 0.1 M NaCl–0.05 M Tris [pH 7.6]–0.005 M KCl) was formed above these. Discontinuous gradients were formed by making the suspension 45% with respect to sucrose and overlaying with 3.4 ml of 35% sucrose and 0.2 ml of buffer (0.1 M NaCl–0.05 M Tris [pH 7.6]–0.005 M KCl). Gradients were centrifuged in an SW 56 rotor for 17 h at 45,000 rpm at 4°C. Tubes were pierced, and fractions were collected from below. The lipid vesicles were isolated from the gradients, diluted with buffer, and sedimented at 25,000 rpm in the SW 56 or SW 27 rotor for 2 h at 4°C.

Proteolytic digestion of vesicles. Proteolysis of vesicles was carried out by making the suspension 0.05 M with respect to ammonium bicarbonate (pH 8.6) and adding 40 μg of TPCK trypsin (Worthington Biochemicals Corp.) or 40 μg of thermolysin (Calbiochem) and incubating for 1 h at 37°C. Samples were then made 45% with respect to sucrose, overlaid with 35% sucrose and 0.2 ml of buffer (0.1 M NaCl, 0.05 M Tris [pH 7.6], 0.005 M KCl) and centrifuged at 45,000 rpm for 17 h at 4°C in the SW 56 rotor. The vesicles at the top of the gradient were diluted with buffer and sedimented at 25,000 rpm for 2 h in the SW 27 rotor.

Mixing of M or tryptic fragments of M with preformed vesicles. The ability of M to associate with preformed vesicles was determined. M was extracted with acidic chloroform-methanol, and the organic solvent was removed by vacuum. The protein was used as such or was solubilized with 1% deoxycholate in 0.1 M NaCl–0.05 M Tris (pH 7.6)–0.005 M KCl. The detergent was dialyzed against several changes of the same buffer containing 0.002 M NaN_3 for 36 h.

M which had been solubilized with deoxycholate and then dialyzed was digested with trypsin for 2 h at 37°C at a concentration of 10 to 20% trypsin.

Vesicles were made by drying the lipid by vacuum and suspending it in buffer containing 1% deoxycholate. Detergent was removed by dialysis, and the vesicles were then added to dried M, to M which had been solubilized with deoxycholate and the detergent dialyzed out, or to tryptic digests of M. The lipid/protein ratio was kept at 10:1. Samples were agitated for 5 min in the Vortex mixer, made dense with sucrose, and floated upward through 35% sucrose as outlined above.

Polyacrylamide gel analysis. The pellets of vesicles or other samples were solubilized with sodium dodecyl sulfate and mercaptoethanol and analyzed on 12% sodium dodecyl sulfate-polyacrylamide gels as previously described (38). The gels were frozen and cut into 1-mm slices; the material was eluted by shaking the slices overnight in 0.4 ml of water. Radioactivity was determined after addition of toluene-based scintillation fluid in a Nuclear Chicago scintillation counter. Molecular weight was determined by running WSN virus, cytochrome *c*, and insulin (Schwarz/Mann) in parallel on the same slab gel. The gel was stained with 1% Coomassie blue in 10% trichloroacetic acid–10% isopropyl alchol and destained in methanol-water-acetic acid (5:5:1).

Two-dimensional analysis on silica gel plates. Tryptic digests were suspended in pyridine-acetic acid-water (162:14:824), spotted on silica HL plates (Analtech, Inc.), and electrophoresed in the same solvent at 300 V as previously described (12). Chromatography was with pyridine-acetic acid-water-butanol (20:6:10:30). Further analysis of the fragment associated with lipid vesicles was carried out by two-dimensional chromatography, the first in pyridine-acetic acid-water-butanol (20:6:10:30) and the second direction in phenol-formic acid-water-butanol (20:5:10:2).

Electrophoresis of cyanogen bromide fragments of M was with formic acid-acetic acid-water (348:100:552). Chromatography was in pyridine-acetic acid-water-butanol (20:6:10:30).

Cleavage of M with cyanogen bromide and association of fragments with lipid vesicles. M was extracted from virus with chloroform-methanol, precipitated with 5 volumes of cold ether, collected by centrifugation, and suspended in 75% formic acid. Cyanogen bromide (Pierce Chemical Co.) was added at 70-fold excess and allowed to react for 17 h at room temperature. Cyanogen bromide was removed by blowing air into the tube, and the material was dried by lyophilization and suspended in 0.1 M NaCl–0.05 M Tris (pH 7.6)–0.005 M KCl–1% deoxycholate and dried phosphatidylcholine. Detergent was removed by dialysis as described above. Samples were made dense with sucrose, and vesicles floated upward through 35% sucrose.

Association of cyanogen bromide fragments with lipid was also carried out by removing the cyanogen

bromide with a stream of air and then taking the solution of fragments in 75% formic acid and adding this to dried lipid. The acid was then dialyzed out against 0.1 M NaCl-0.05 M Tris (pH 7.6)-0.005 M KCl.

Amino acid analysis. The amino acid composition of the fragment associated with trypsinized vesicles was determined with a Beckman model 120C amino acid analyzer. Vesicles containing M were trypsinized with excess trypsin (50%) for 2 h at 37°C, separated by flotation as described above, and pelleted. The lipid was removed by extracting the pellet twice with 2 ml of methanol followed by one extraction with chloroform. The protein was sedimented each time at 20,000 rpm for 10 min and finally hydrolyzed with 6 N HCl containing 1% mercaptoethanol for 20 h at 110°C.

RESULTS

Association of M with lipid vesicles. The possible interaction of M with the lipid of the virus was investigated by studying the behavior of this protein in vitro with vesicles of viral lipid or egg phosphatidylcholine. The protein was mixed with lipid, and vesicles were formed (i) by using deoxycholate followed by dialysis or (ii) by sonication. The association of M with the lipid vesicles was then determined by floating the suspension through 5 to 30% sucrose gradients. Figure 1 shows the results of an experiment in which lipid vesicles were formed with M by the detergent dialysis method. On centrifugation, M protein alone remained at the bottom of the tube (Fig. 1A). Viral lipid or phosphatidylcholine alone floated towards the top of the gradient, and both had a pattern similar to that depicted for phosphatidylcholine in Fig. 1B. When M was mixed with viral lipid (Fig. 1C) or egg phosphatidylcholine (Fig. 1D), M and the lipid floated upward. The density of phosphatidylcholine vesicles was 1.06 g/ml whereas the vesicles formed between M and phosphatidylcholine were more dense and peaked at 1.10 g/ml. The density of M and viral lipid was 1.07 g/ml. Between 90 and 100% of the M protein associated with lipid vesicles and floated upward after centrifugation, regardless of the method used to purify M and irrespective of whether vesicles were formed by detergent dialysis or by sonication. However, because the yields of M were better, the chloroform-methanol method was most often used to purify the protein from the virus. Flotation patterns were unaffected by the incorporation of 0.5 M NaCl into the gradients to eliminate electrostatic interactions.

The formation of vesicles after mixing of M with lipid was corroborated by examining preparations under the electron microscope. The vesicles formed with viral lipid ranged from 30 to 120 nm in diameter, whereas those formed between M and phosphatidylcholine were some-

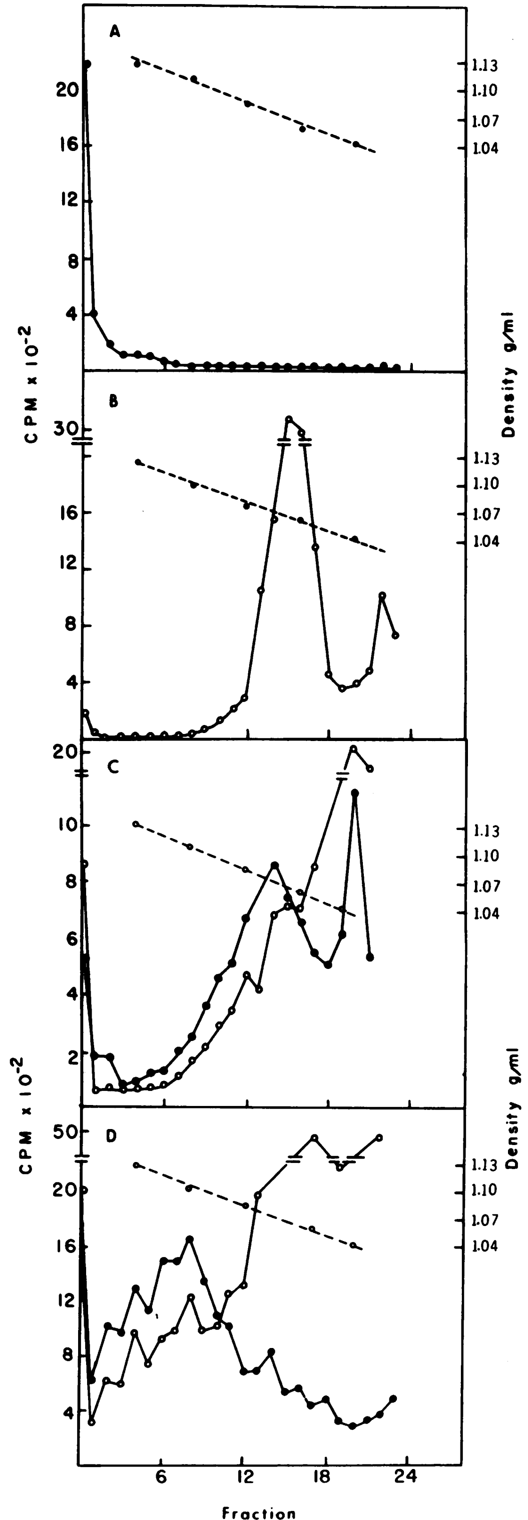


FIG. 1. *Flotation analysis of M mixed with lipid through continuous gradients. [^{35}S]methionine-la-*

what larger, ranging from 70 to 150 nm in diameter.

Studies were also carried out with the ribonucleoprotein of influenza virus to determine if that component could also associate with lipid under similar conditions. The ribonucleoprotein was isolated by the method of Pons et al. (29), and the solution was added to dried phosphatidylcholine. The material was made 1% with respect to deoxycholate and mixed for 5 min; the detergent was then removed by dialysis. The material was analyzed by flotation through 35% sucrose as outlined above for M. All of the nucleoprotein remained at the bottom of the gradient; thus, no association occurred between nucleoprotein and lipid. Under similar conditions, at least 95% of the M protein attached to lipid and floated upward.

Incorporation of M into preformed lipid vesicles. The ability of M to interact with preformed vesicles was also investigated. Preformed phosphatidylcholine vesicles were mixed with either dried M or with M which had been solubilized with deoxycholate and the detergent then removed by dialysis. The vesicles and protein were mixed for 5 min in a Vortex mixer, and the samples were analyzed by flotation through discontinuous sucrose gradients. Approximately 65% of the M in buffer solution associated with the lipid, as judged by its position in the gradient (Fig. 2B). When M was completely dry, approximately 55% of the protein associated with the lipid to float to the top of the gradient (Fig. 2C). The ability of M to float upward through 35% sucrose against a centrifugal force of over 100,000 × *g*, even in the presence of 0.5 M NaCl, which would eliminate electrostatic forces, suggested that the protein was tightly associated with the lipid vesicles.

Thermolysin and trypsin treatment of vesicles containing M. The association of M with lipid vesicles was further characterized by exposing these vesicles to trypsin or thermolysin. Vesicles containing M formed by sonication were exposed to trypsin or thermolysin for 1 h at 37°C, and vesicles floated upward through discontinuous gradients. Fractions were collected from below and assayed for radioactivity. The radioactivity which was associated with lipid vesicles that were untreated with enzyme floated to the top of the gradient (Fig. 3A). After treatment with either trypsin (Fig. 3B) or thermolysin (Fig. 3C), approximately 80 to 85% of the radioactivity was removed from the vesicles and remained at the bottom of the tube, whereas the rest of the radioactivity was associated with the vesicles at the top of the gradients. The results

beled M was mixed with lipid and deoxycholate. The detergent was removed by dialysis and the samples were made 45% with respect to sucrose and floated through 5 to 30% sucrose gradients. Trace amounts of [^{3}H]phosphatidylcholine were added as marker. ●, [^{35}S]methionine-labeled M; ○, [^{3}H]phosphatidylcholine. (A) M alone; (B) egg phosphatidylcholine alone; (C) M mixed with viral lipid; (D) M mixed with egg phosphatidylcholine.

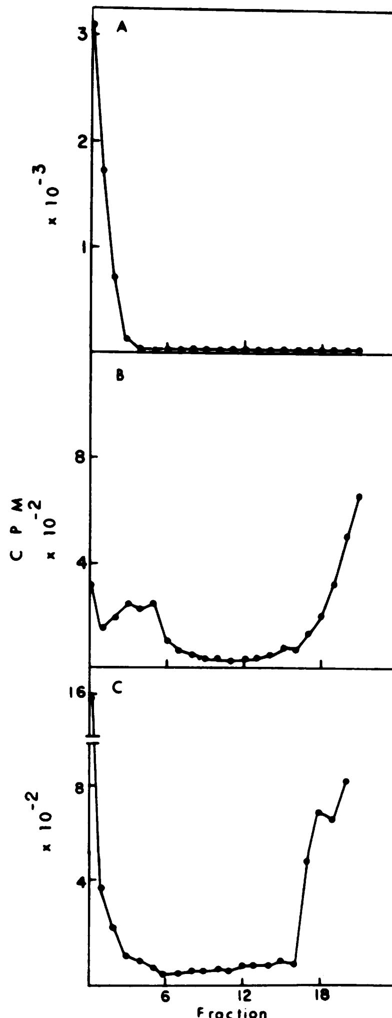


FIG. 2. *Discontinuous gradients of M mixed with preformed vesicles. Phosphatidylcholine vesicles were formed by detergent dialysis and added to M protein. Samples were agitated on a Vortex mixer for 5 min, made 45% with respect to sucrose, and analyzed by flotation through 35% sucrose. (A) M protein alone. The protein was extracted with chloroform-methanol, dried, and suspended in buffer containing 1% deoxycholate. The detergent was then removed by dialysis. (B) M protein prepared as in A, mixed with preformed lipid vesicles. (C) Dried M mixed with preformed vesicles. M was extracted from virus with chloroform-methanol and dried by vacuum in this case. M was labeled in [^{35}S]methionine.*

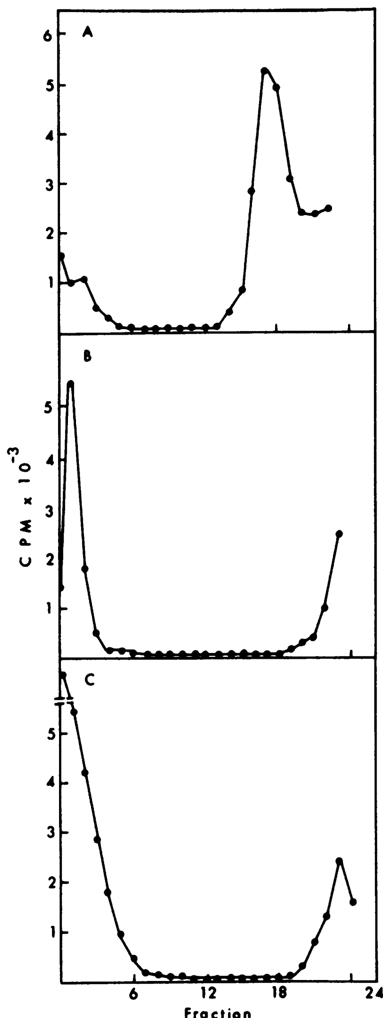


FIG. 3. *Discontinuous gradients of trypsin- or thermolysin-treated vesicles containing M. ^{3}H-amino acid-labeled M was extracted with choloroform-methanol, mixed with phophatidylcholine, and sonicated. Suspensions were treated with trypsin or thermolysin for 1 h at 37°C and analyzed by flotation through 35% sucrose. (A) Untreated vesicles containing M, (B) trypsin-treated vesicles containing M, (C) thermolysin-treated vesicles containing M.*

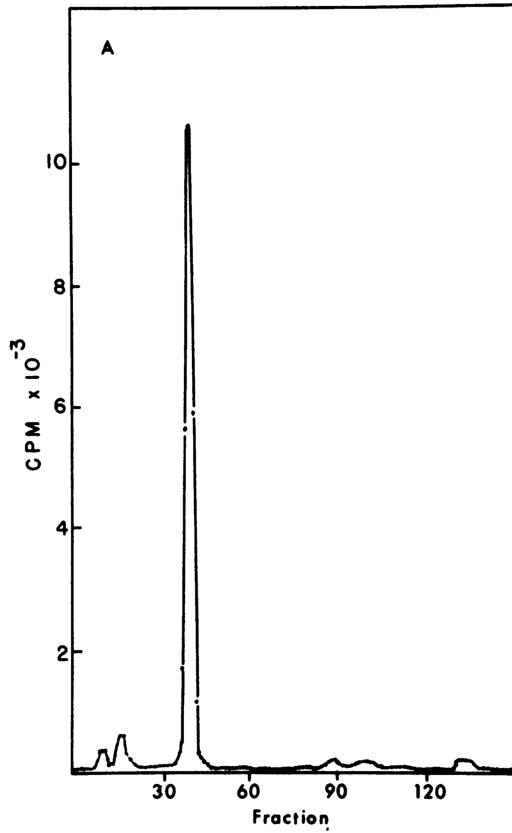
of proteolytic digestion of vesicles which were prepared by mixing M with preformed vesicles were identical to those shown in Fig. 3B and C.

The ability of tryptic fragments of M to associate with vesicles was also investigated. M was extracted from the virus, and the protein was solubilized with deoxycholate. The detergent was removed by dialysis, and the protein was trypsinized. Preformed vesicles were added, and the association of fragments to vesicles was determined by flotation after the samples were blended in a Vortex mixer for 5 min. Using these conditions, the tryptic fragments of M remained at the bottom of the tube. Tryptic fragments did not associate with preformed vesicles to any substantial degree.

Analysis of vesicles by polyacrylamide gel electrophoresis. The vesicles which floated upward after proteolytic digestion were analyzed for protein composition. Lipid vesicles at the top of gradients as depicted in Fig. 3 were pooled, sedimented, solubilized with sodium dodecyl sulfate and mercaptoethanol, and analyzed on polyacrylamide gels. Untreated vesicles contained M only (Fig. 4A). Small amounts of material larger than 25,000 daltons (fractions 10 to 15) were aggregates appearing between the 4% spacer and the 12% running gel. Vesicles treated with trypsin (Fig. 4B) or thermolysin (Fig. 4C) contained some undigested M and a smaller fragment appeared which was resistant to proteolysis (fraction 100). If excess amounts of trypsin or thermolysin (50%) were used, all of the original M was hydrolyzed, except for the smaller fragment (Fig. 4D). The apparent molecular weight of the fragment was approximately 5,000. If vesicles containing M were first disrupted by adding 1% deoxycholate and trypsin or thermolysin was then added, fragments of various sizes were produced which were both larger and smaller than the 5,000-dalton component (data not shown).

Two-dimensional analysis of trypsinized vesicles on silica gels. Trypsinized vesicles were further analyzed by two-dimensional separation on silica gel plates. Vesicles containing M which had been treated with excess trypsin for 2 h were separated from tryptic fragments by flotation, solubilized in pyridine-acetic acid-water, and analyzed in two dimensions on silica gel plates. The total tryptic digest of vesicles which contained M is shown in Fig. 5A. After trypsinization, one fragment remained associated with vesicles (Fig. 5B). This peptide remained at the origin and did not migrate in either direction

FIG. 4. *Polyacrylamide gel electrophoresis of vesicles containing M after incubation with proteolytic enzymes. M was reacted with lipid by the detergent dialysis method and vesicles were treated with enzyme. Vesicles were then isolated by flotation, solubilized with sodium dodecyl sulfate and mercaptoethanol, and analyzed on 12% polyacrylamide gels. (A) Untreated vesicles containing M, (B) trypsin-treated vesicles, (C) thermolysin-treated vesicles, (D) vesicles treated with excess (50%) concentration of trypsin. Migration is towards the right. M was labeled with a mixture of ^{3}H-amino acids.*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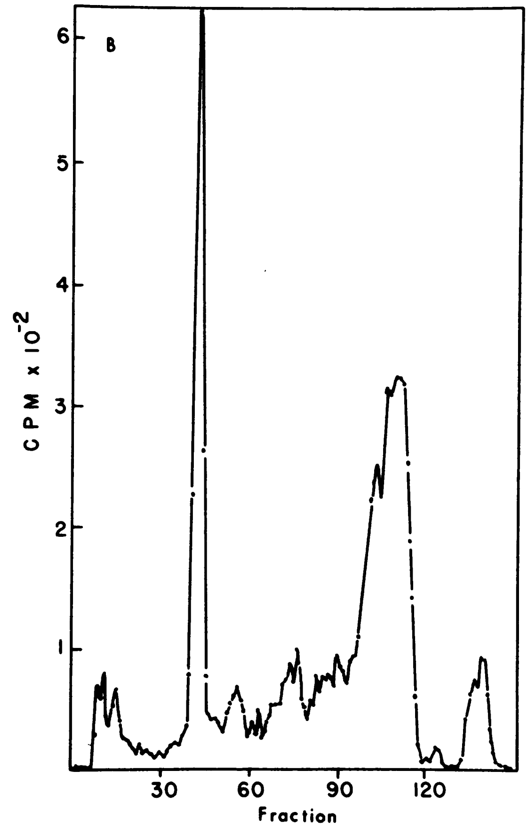
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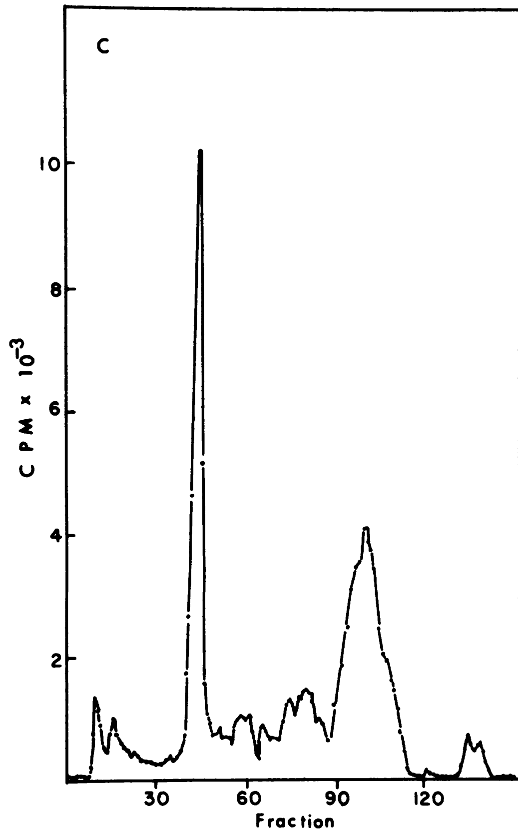
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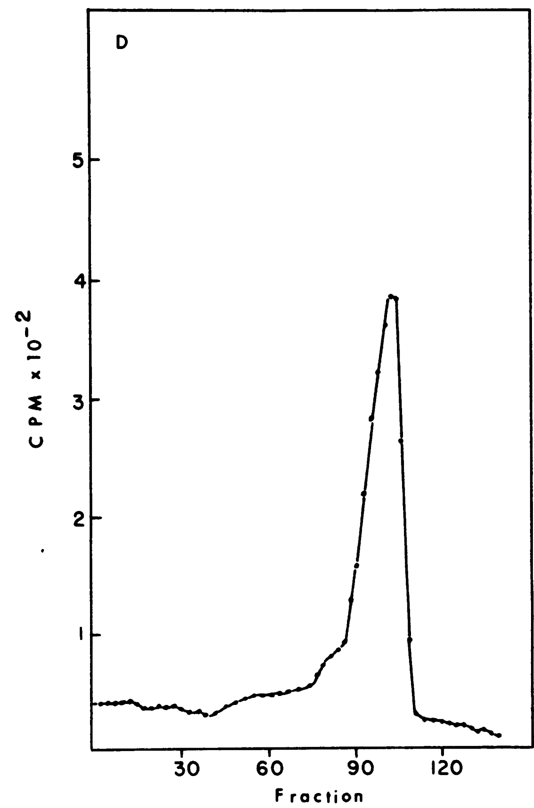
D
CPM x 10^-2
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under the conditions specified above. Migration of the fragment away from the origin was possible by chromatographing in pyridine-acetic acid-water-butanol (20:6:10:30) in the first direction (I) and in phenol-formic acid-water-butanol (20:5:10:2) in the second direction (II), as shown in Fig. 5C. The fragment was very insoluble and aggregated in aqueous buffers once the lipid was removed, so that trypsin and chymotrypsin were ineffective in digesting it further.

Association of cyanogen bromide fragments of M with lipid. The data thus far suggested that a specific part of the M protein interacted with lipid vesicles in vitro. This was further tested by taking cyanogen bromide fragments of M and mixing these with lipid as described above. Vesicles were harvested after flotation through 35% sucrose and analyzed in two dimensions on silica HL plates. At least 13 fragments were produced when M was cleaved at the methionine residues by cyanogen bromide (Fig. 6A). Of these 13, 3 specifically bound tightly to lipid to float upward through discontinuous gradients (Fig. 6B). It is likely that these cyanogen bromide fragments represent part of the same area of the M molecule which remained associated with vesicles after the extensive trypsinization discussed above. This possibility is presently being investigated.

Amino acid composition of the lipid-associated fragment. The amino acid composition of the fragment of the M protein remaining

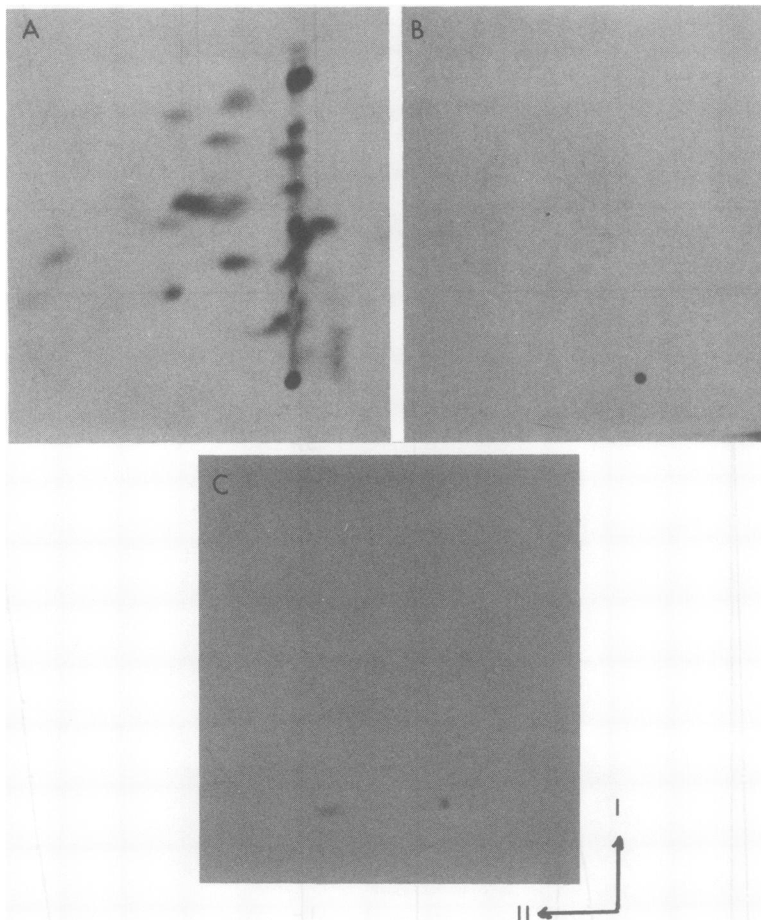

FIG. 5. *Two-dimensional analysis on silica gel plates of fragment associated with trypsinized vesicles. (A) Total tryptic digest of vesicles containing M labeled in ^{3}H-amino acids; (B) vesicles after excess trypsinization and flotation. A and B were electrophoresed in pyridine-acetic acid-water (162:14:824) and chromatographed in pyridine-acetic acid-water-butanol (20:6:10:30). (C) Two-dimensional chromatography of vesicles after excessive trypsinization. Dimension I, pyridine-acetic acid-water-butanol (20:6:10:30); dimension II, phenol-formic acid-water-butanol (20:5:10:2).*

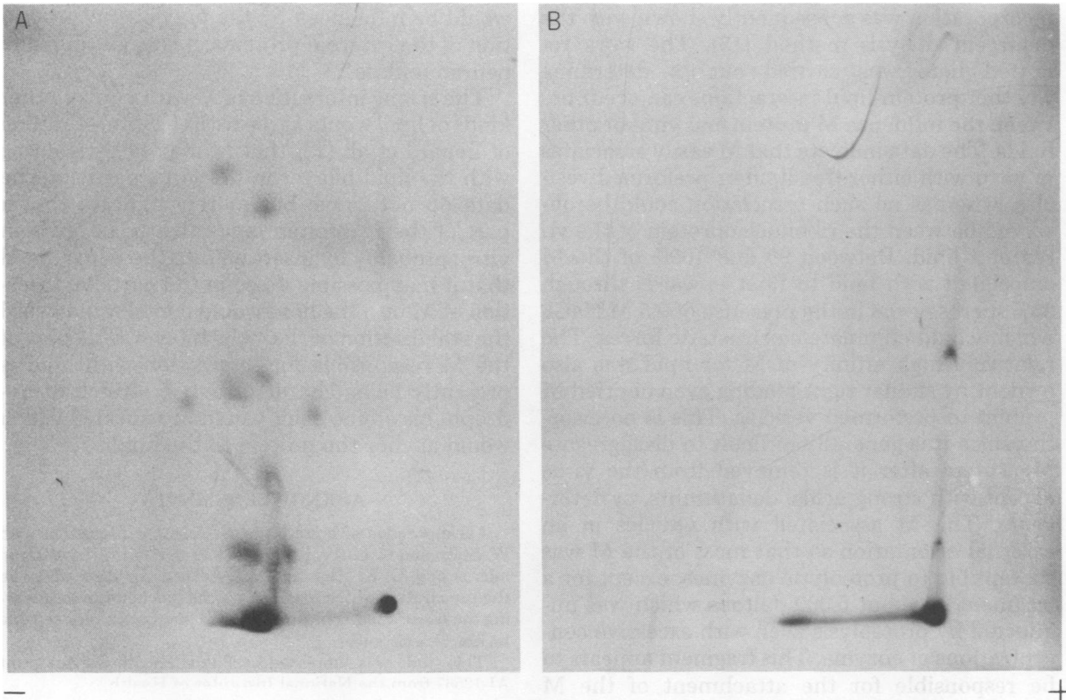

FIG. 6. *Two-dimensional analysis of cyanogen bromide fragments of M which floated upwards through 35% sucrose with lipid vesicles. (A) Total mixture containing cyanogen bromide fragments and phosphatidylcholine; (B) fragments associated with vesicles at the top of the gradient after flotation. M protein had been labeled with a mixture of* ^{14}C*-amino acids.*

on trypsinized vesicles was determined. Trypsinized vesicles were separated from tryptic peptides by flotation and pelleted; the lipid was removed with methanol and chloroform as described above. The amino acid composition of the fragment is given in Table 1. The fragment contained 24% charged amino acids, while the rest could be considered hydrophobic (39). For comparison, Table 1 also lists the bromelain fragment of the hemagglutinin which remains embedded into the lipid of the virus (36).

DISCUSSION

Artificial membrane vesicles have been widely used in studies pertaining to biological membrane systems and to membrane biogenesis. Reconstitution experiments which began with mitochondrial proteins (19) have also been carried out with cell surface antigens (10, 23) and surface components of Sendai virus (16, 17), influenza virus (1, 18), Semliki Forest virus (15), retroviruses (33), and vesicular stomatitis virus (28). The data involving cellular and viral components have indicated that the proteins insert themselves into the liposomes in an orientation identical to that found in the normal host.

The incorporation of the hemagglutinin and

TABLE 1. *Estimated amino acid residues*

Amino acid	M Fragment	Hemagglutinin fragment[a]
Lysine	2	2
Histidine	1	0
Argenine	1	1
Aspartic acid	4	4
Threonine	4	2
Serine	9	11
Glutamic acid	4	6
Proline	2	2
Glycine	8	5
Alanine	6	3
Valine	2	5
Cysteine	0	2
Methionine	2	1
Isoleucine	2	1
Leucine	4	8
Tyrosine	1	1
Phenylalanine	1	1

[a] Data of Skehel and Waterfield (36).

neuraminidase of influenza into lipid vesicles of defined composition was shown by Almeida et al. (1) when they sonicated these proteins with preformed vesicles. "Virosomes" were produced which had spike structures identical in appearance to those of whole virus. The same type of

incorporation was subsequently shown with the detergent dialysis method (18). The work reported here was carried out to determine whether protein-lipid interactions can occur between the influenza M protein and viral or other lipids. The data indicate that M easily associates in vitro with either free lipid or preformed vesicles, whereas no such association could be observed between the ribonucleoprotein of the virus and lipid. Between 90 and 100% of the M associated with lipid to float upwards through 35% sucrose even in the presence of 0.5 M NaCl, which would eliminate electrostatic forces. The relatively high affinity of M for lipid was also evident by similar tight binding even of dried M protein to preformed vesicles. This is noteworthy since it is generally difficult to disaggregate M protein after it is removed from the virus except with strong acids, denaturants, or detergents. The M associated with vesicles in an external orientation so that most of the M was susceptible to proteolytic enzymes, except for a fragment of about 5,000 daltons which was unaffected by proteolysis even with excessive concentrations of enzyme. This fragment appears to be responsible for the attachment of the M protein to the vesicles and represents a specific part of the molecule. That a certain area of the molecule can interact with lipid is further suggested by the finding that 3 cyanogen bromide fragments of the 13 produced bound tightly specifically to lipid vesicles. These fragments are being separated and will be analyzed to determine whether they are part of the same area of the molecule represented by the 5,000-dalton fragment discussed above.

Most of the evidence available (with one exception; see reference 30) indicates that M is below the lipid bilayer of the virus particle. Proteolytic enzymes which remove the hemagglutinin and neuraminidase from whole virus do not affect the M or the nucleoprotein (9, 34), nor can the protein be detected on the surface of the virus with antibody to M (27) or by labeling the surface proteins with radioactive groups (31, 37). It can, nevertheless, appear on the surface of the infected cell, where it accumulates by an active process (4, 6). The process of assembly of this protein thus seems unclear, since it can be found externally on the surface of the cell where budding occurs. The protein appears in the cytoplasm in insoluble form (14, 26) and in the nucleus, where large amounts accumulate even early after infection (12, 14, 24). The finding here that M orients itself externally on lipid vesicles is not surprising, since the experiments were carried out with purified M. The positioning at the actual site of budding in the infected cell would be influenced by the previous incorporation of the external proteins, hemagglutinin and neuraminidase.

The strong interaction of M with viral or other kinds of lipid would agree with the interpretation of Lenard et al. (22) that M may be interacting with the lipid bilayer in the virus particle. The data do not prove but merely indicate that a part of the M protein binds tightly to lipids in vitro, probably by insertion into the bilayer, and that it may possibly do so in the particle. Insertion of M into the lipid would contribute toward the stabilization of the virus bilayer. The part of the M responsible for interaction with lipid is presently being characterized. A stretch of hydrophobic amino acids would be expected which would anchor the protein to the lipid.

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