Hitchcock, S. E. (1975) *Biochemistry 14*, 5162–5167.
Hudson, E. N., & Weber, G. (1976) *Biochemistry 15*, 672–680.
Isenberg, I., & Dyson, R. D. (1969) *Biophys. J. 9*, 1337–1350.
Isenberg, I., Dyson, R. D., & Hanson, R. (1973) *Biophys. J. 13*, 1090–1115.
Kawasaki, Y., & van Eerd, J. P. (1972) *Biochem. Biophys. Res. Commun. 49*, 898–905.
Lamkin, M., & Tao, T. (1982) *Biophys. J. 37*, 37a.
Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem. 253*, 5452–5459.
Leavis, P. C., Gowell, E., & Tao, T. (1984) *Biophys. J. 45*, 262a.
Lehrer, S. S., & Leavis, P. C. (1978) *Methods Enzymol. 49*, 222–236.
Levine, B. A., Thornton, J. M., Fernandes, J. M., Kelly, C. M., & Mercola, D. (1978) *Biochim. Biophys. Acta 535*, 11–24.
Margossian, S. S., & Cohen, C. (1973) *J. Mol. Biol. 81*, 409–413.
Morris, E., & Lehrer, S. S. (1984) *Biochemistry 23*, 2214–2220.
Nagy, B., Potter, J. D., & Gergely, J. (1978) *J. Biol. Chem. 253*, 5471–5474.
Ohnishi, S., Maruyama, K., & Ebashi, S. (1975) *J. Biochem. (Tokyo) 78*, 73–81.
Potter, J. D., & Gergely, J. (1974) *Biochemistry 13*, 2697–2703.
Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628–4633.
Potter, J. D., Seidel, J. C., Leavis, P. C., Lehrer, S. S., & Gergely, J. (1976) *J. Biol. Chem. 251*, 7551–7556.
Seamon, K. B., Hartshorne, D. J., & Bothner-By, A. (1977) *Biochemistry 16*, 4039–4046.
Small, E., & Isenberg, I. (1976) *Biopolymers 15*, 1093–1100.
Small, E., & Isenberg, I. (1977) *J. Chem. Phys. 66*, 3347–3351.
Tao, T., & Cho, J. (1979) *Biochemistry 18*, 2759–2765.
Tao, T., Lamkin, M., & Scheiner, C. (1984) *Biophys. J. 45*, 261a.
Weeks, R. A., & Perry, S. V. (1978) *Biochem. J. 173*, 449–457.
Zot, H. G., & Potter, J. D. (1982) *J. Biol. Chem. 257*, 7678–7683.

Effect of Lipid Composition upon Fusion of Liposomes with Sendai Virus Membranes†

Anne M. Haywood* and Bradley P. Boyer

ABSTRACT: How the lipid composition of liposomes determines their ability to fuse with Sendai virus membranes was tested. Liposomes were made of compositions designed to test postulated mechanisms of membrane fusion that require specific lipids. Fusion does not require the presence of lipids that can form micelles such as gangliosides or lipids that can undergo lamellar to hexagonal phase transitions such as phosphatidylethanolamine (PE), nor is a phosphatidylinositol (PI) to phosphatidic acid (PA) conversion required, since fusion occurs with liposomes containing phosphatidylcholine (PC) and any one of many different negatively charged lipids such as gangliosides, phosphatidylserine (PS), phosphatidylglycerol, dicetyl phosphate, PI, or PA. A negatively charged lipid is required since fusion does not occur with neutral liposomes containing PC and a neutral lipid such as globoside, sphingomyelin, or PE. Fusion of Sendai virus membranes with liposomes that contain PC and PS does not require Ca^{2+}, so an anhydrous complex with Ca^{2+} or a Ca^{2+}-induced lateral phase separation is not required although the possibility remains that viral binding causes a lateral phase separation. Sendai virus membranes can fuse with liposomes containing only PS, so a packing defect between domains of two different lipids is not required. The concentration of PS required for fusion to occur is approximately 10-fold higher than that required for ganglioside G_{D1a}, which has been shown to act as a Sendai virus receptor. When cholesterol is added as a third lipid to liposomes containing PC and G_{D1a}, the amount of fusion decreases if the G_{D1a} concentration is low. This suggests cholesterol may decrease the availability of G_{D1a} for multivalent binding. These data are consistent with the postulate that viral binding and the activities of the viral proteins play the major role in the fusion of viral and liposomal membranes.

Many hypotheses about the mechanism of membrane fusion involve properties of the membrane that result from specific lipids present in the membrane. These hypotheses include local micellization (Lucy, 1970) which could result from aggregation of gangliosides (Haywood, 1974a), lamellar to hexagonal phase transitions or formation of inverted micelles (Cullis & de Kruijff, 1979), packing defects due to transitions between domains of individual lipids with different packing packing properties or to lateral phase separations such as that due to the binding of Ca^{2+} to negatively charged lipids (Papahadjopoulos, 1978). These hypotheses also include the metabolic conversion of phosphatidylinositol (PI) to phosphatidic acid (PA) or changes in membrane hydration due to anhydrous Ca^{2+} complexes (Portis et al., 1979) or to the varying affinities of different lipids for water (Jendrasiak & Hasty, 1974).

Most of these hypotheses have been tested by examining fusion between two model membranes and may not reflect the mechanism of fusion of biologic membranes where membrane

† From the Departments of Pediatrics and Microbiology, University of Rochester Medical Center, Rochester, New York 14642. *Received January 4, 1984.* This work was supported by U.S. Public Health Service Research Grant AI-15540 and by National Science Foundation Grant PCM-8205896.

* Address correspondence to this author at the Department of Pediatrics, University of Rochester Medical Center.

0006-2960/84/0423-4161$01.50/0

proteins play a role in the fusion event. Previously it has been shown that Sendai virus membranes fuse with liposomes in a manner similar to the way they fuse with cellular membranes (Haywood, 1974b; Haywood & Boyer, 1981) and that the viral proteins appear to control the fusion with liposomes (Haywood & Boyer, 1982). The Sendai virus–liposome system therefore makes it possible to determine the effect of liposomal lipid composition in a fusion event determined by a biologic membrane. In this work, the liposomal lipid composition is varied in a manner to test the different hypotheses of membrane fusion that involve specific compositions.

Experimental Procedures

Buffers. PBS contains 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 with the pH adjusted to 7.5. For experiments where the pH was to be varied, virus was dialyzed against "low phosphate" PBS containing $^1/_{100}$th the concentration of phosphate, i.e., 0.08 mM Na_2HPO_4 and 0.015 mM KH_2PO_4. HKN contains 130 mM NaCl, 2.7 mM KCl, and 3 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) with the pH adjusted to 7.5.

Virus. Virus was grown and radiolabeled in embryonated eggs, purified, and dialyzed against PBS as previously described (Haywood & Boyer, 1982). The protein content of the virus preparation was measured by a modification of the Lowry procedure (Markwell et al., 1978). Three different virus preparations were used and contained 698 cpm/μg of protein (Table I), 829 cpm/μg of protein (Figures 1–3, Table II), and 2000 cpm/μg of protein (Tables III and IV).

Lipids. Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), egg phosphatidic acid (PA), bovine brain sphingomyelin, bovine brain phosphatidylserine (PS), and bovine brain phosphatidylinositol (PI) were obtained from Avanti Biochemicals, Inc. Globoside and phosphatidylglycerol (PG) were obtained from Supelco, Inc. The PG was further purified by thin-layer chromatography using chloroform/methanol/acetic acid/water (65:25:2:4 v/v) and silica gel G plates. Dicetyl phosphate and cholesterol, chromatography standard grade, were obtained from Sigma Chemical Co. When 0.8 μmol of these lipids was chromatographed with chloroform/methanol/concentrated NH_4OH (60:25:4 v/v) and with chloroform/methanol/acetic acid/water (65:25:2:4 v/v) on silica gel G thin-layer chromatography plates, only one spot was observed. Ganglioside G_{D1a} was also obtained from Supelco, Inc. Only one spot was obtained when 0.3 μmol was chromatographed with chloroform/methanol/2.5 M NH_4OH (60:35:8 v/v) plus 20 mg of KCl/100 mL. The concentrations of the phospholipids were determined by the phosphate assay of Ames & Dubin (1960), the concentration of G_{D1a} by the sialic acid assay of Miettinen & Takki-Luukkainen (1959), and the cholesterol concentration by the assay of Courchaine et al. (1959).

Formation and Fusion of Liposomes. Multilamellar liposomes were made in PBS as previously described (Haywood & Boyer, 1981). Virus and liposomes in a final volume of 0.3 mL were incubated for 2 h at 40 °C unless stated otherwise.

Electron Microscopy. Samples were diluted through 0.145 M ammonium acetate, stained with 2% ammonium molybdate, pH 7.1, and examined in the electron microscope as previously described (Haywood & Boyer, 1981).

Fusion Assay. The amount of fusion was quantitated by measuring the amount of [^{35}S]methionine-labeled viral protein incorporated into liposomes during fusion. The viruses that were bound to liposomes but not fused were separated from the fused liposomes by layering under a discontinuous sucrose gradient and centrifuging so the fused liposomes rose to the top of the gradient as previously described (Haywood & Boyer, 1982). In some experiments, an SW60 rotor rather than an SW65 rotor was used, and the samples were centrifuged at 54 000 rpm for 40 min. To be sure the liposomal composition did not affect the fusion assay, the amount of labeled viral protein that remained associated with liposomes was assayed for liposomes of each composition after the liposomes and virus had been kept at 0 °C for 1 h. Under these conditions, which should allow binding but not fusion, between 3 and 4% of the total counts added remained with the liposomes for all liposomal compositions. Therefore, in measurement of fusion, a background of 3.5% of the total counts added was subtracted from the counts associated with the liposomes.

Table I: Effect of Preadsorption of Virus at 0 °C upon Subsequent Membrane Fusion[a]

min at 40 °C	viral protein (cpm) incorp into liposomes: with preadsorption	viral protein (cpm) incorp into liposomes: without preadsorption
30	438	493
120	881	980

[a] Liposomes were made from 0.7 μmol of PC, 0.3 μmol of PE, 0.66 μmol of cholesterol, and 0.03 μmol of ganglioside G_{D1a} in 0.15 mL of PBS. ^{35}S-Labeled virus (5000 cpm) in 0.15 mL of PBS was added and either preadsorbed for 1 h in an ice bucket and then transferred to a 40 °C water bath or put directly at 40 °C. Samples were removed after 30 and 120 min at 40 °C.

Results and Discussion

Effect of Preadsorption of Virus to Liposomes at 0 °C. Paramyxoviruses and influenza virus bind to host cells, red cells, and ganglioside-containing liposomes in the cold (0–4 °C), but the virus does not penetrate cells or liposomes until the temperature is raised. Since viral sialidases are active at higher temperatures, studies of these viruses have conventionally been carried out by adsorbing the viruses to cells in the cold and then raising the temperature to initiate entry. This convention was followed in the previous studies of Sendai virus with liposomes (Haywood, 1974a; Haywood & Boyer, 1982). Clearly this procedure is not biologic and bears no resemblance to events in natural infection. Furthermore, these changes of temperature introduce changes in the physical state of the virus and liposome which could complicate interpretation of events. Therefore, the effect of preadsorption of virus in the cold upon the amount of fusion that occurred during a subsequent incubation at 40 °C was tested. As shown in Table I, the amount of fusion that occurs both after 30 min at 40 °C and after 120 min at 40 °C is independent of whether the virus was preadsorbed to liposomes for 1 h in an ice bucket. Therefore, for subsequent studies, the preadsorption step was omitted, and the virus and liposomes were mixed and directly incubated at 40 °C. This result differs from the observation of Lyles & Landsberger (1979), who found that the amount of spin-label transferred from virus to red cells was decreased if the virus was not preattached in the cold. This difference could be either because the red cell receptor behaves differently from G_{D1a} or because Lyles and Landsberger were measuring phospholipid transfer which includes nonspecific transfer as well as transfer due to fusion.

Virus Membrane Fusion with Liposomes Containing Only PC and Ganglioside. Viruses were previously shown to be able to fuse their membranes with liposomes made from 0.7 μmol of PC, 0.3 μmol of PE, 0.66 μmol of cholesterol, and the ganglioside G_{D1a} (Haywood & Boyer, 1981). The ganglioside had to be included in the composition for fusion to occur, and as the mole percent of G_{D1a} increased, the amount of fusion

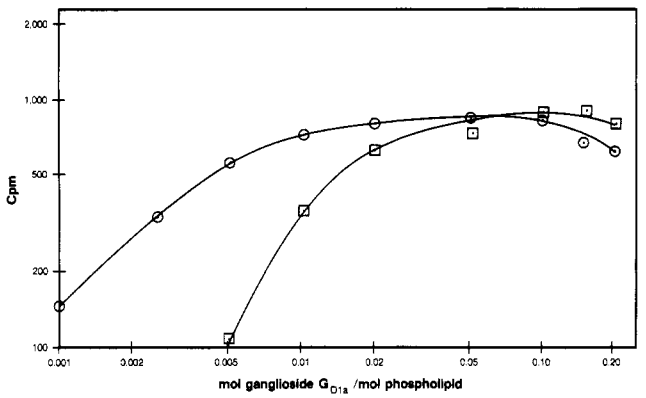

FIGURE 1: Fusion of Sendai virus membranes with liposomes containing PC and varying concentrations of G_{D1a} with or without cholesterol. Liposomes were made from 1 μmol of PC and varying amounts of G_{D1a} or from 1 μmol of PC, 0.66 μmol of cholesterol, and varying amounts of G_{D1a}. ^{35}S-Labeled Sendai virus (5000 cpm) was added and incubated for 2 h at 40 °C, and the amount of labeled viral protein incorporated into liposomes was counted. (○) PC and gangliosides; (□) PC, gangliosides, and cholesterol.

Table II: Effect of Cholesterol upon Fusion of Liposomes Containing a Suboptimal Amount of Receptor[a]

cholesterol (mol %)	viral proteins (cpm) incorp into liposomes	cholesterol (mol %)	viral proteins (cpm) incorp into liposomes
0	461	33	232
17	441	38	204
23	383	40	232
29	349		

[a] Liposomes were made from 1 μmol of PC and 0.005 μmol of G_{D1a} plus different amounts of cholesterol. ^{35}S-Labeled Sendai virus (5000 cpm) was added and incubated for 2 h at 40 °C.

increased until G_{D1a} went above 2 mol % and the amount of fusion reached a plateau. To see if the liposomal composition could be further simplified, virus was incubated with liposomes containing only PC and G_{D1a}. Figure 1 shows the amount of fusion with Sendai virus as a function of G_{D1a} concentration when the only other lipid present is PC. These data differ from the previous data with liposomes that also contained PE and cholesterol in that there is a greater amount of fusion at the low ganglioside concentrations.

Effect of Cholesterol upon Membrane Fusion. Liposomes were made that contained 1 μmol of PC, varying amounts of G_{D1a}, and 40 mol % of cholesterol. Figure 1 shows that the presence of cholesterol in the liposomes results in a decreased amount of fusion at low ganglioside concentrations. At low ganglioside concentrations, the presence of cholesterol decreases the amount of fusion so that it is equivalent to the amount occurring with liposomes containing roughly one-third as much ganglioside and no cholesterol. This suggests that cholesterol may decrease the effective ganglioside concentration. How the decrease in fusion depends upon cholesterol concentration was tested with liposomes made from 1 μmol of PC, 0.05 μmol of G_{D1a}, and varying amounts of cholesterol. As shown in Table II, the amount of fusion slowly decreased as the amount of cholesterol increased up to 33 mol %. It has been suggested that at 33 mol % cholesterol is completely solvated by hydrocarbon chains and that between 33 and 50 mol % cholesterol–cholesterol contacts occur (Engelman & Rothman, 1972).

Since the presence of cholesterol depresses the amount of fusion only at low ganglioside concentrations, it is likely that the effect of cholesterol upon fusion is secondary to a change in the availability of gangliosides. There are a number of ways that cholesterol could change the availability of gangliosides. Addition of cholesterol to egg PC bilayers increases the separation between phospholipids until intermolecular interactions between the phospholipid can no longer occur (Yeagle et al., 1977). By separating the phospholipid head groups, cholesterol may cause the phospholipids to interact more with gangliosides. By changing the membrane fluidity, the presence of cholesterol might decrease the amount of aggregation of gangliosides in response to viral binding.

It has been reported that cholesterol increases the virus-induced fusion between red cells perhaps by facilitating a phase separation of protein-free areas of lipid bilayer (Hope et al., 1977). A requirement for cholesterol has also been reported for Sendai virus induced leakage of a small molecular weight marker from glycophorin-containing liposomes (Kundrot et al., 1983), and the increase in conductance in planar membranes caused by the addition of freeze–thawed Sendai virus is greatly increased if the membranes contain cholesterol (Young et al., 1983). These systems are not directly comparable, since somewhat different phenomena are being measured. The effect produced by cholesterol seems to differ in different situations which is consistent with the idea that cholesterol's effect upon membrane fusion is dependent upon the total composition of the membrane.

Hsu et al. (1983) have studied what they claim to be fusion between sonicated liposomes and Sendai virus and have come to the conclusion that fusion of Sendai virus with liposomes is dependent upon cholesterol but can occur in the absence of gangliosides and other negatively charged lipids. This conclusion is based upon two assays. One assay involved including trypsin in the liposomes and measuring viral protein degradation. More degradation of the viral internal proteins than of the external glycoproteins occurred, but the internal proteins are sensitive to trypsin and the external proteins are relatively resistant. Further, there was no control for leakiness of the membranes, and, since Sendai virus membranes are often leaky and may be even leakier after elastase treatment, such a control is necessary. Their second assay involved transfer of [^{14}C]PC from liposomes to virus, which occurred to a greater extent with virus which contained the mature F protein than with virus which contained the inactive F_0 protein and was therefore unable to fuse. Enhancement of lipid transfer by the F protein does not prove fusion, since Maeda et al. (1977) showed that exchange of spin-labeled PC between Sendai virus and red blood cells without membrane fusion also required active F protein. When [^{35}S]methionine-labeled viruses are incubated for 2 h at 40 °C with sonicated liposomes containing egg PC, ganglioside G_{D1a}, and [^{3}H]glycerol trioleate, which does not undergo lipid exchange (Zilversmit & Hughes, 1976), there is no evidence fusion occurs since there is no mixing of labels (A. M. Haywood and B. P. Boyer, unpublished results). The elastic properties of sonicated vesicles differ from those of larger vesicles, so sonicated vesicles in the fluid phase are not deformable (Evans & Parsegian, 1983). Since Sendai virus membrane fusion involves membrane deformation (Haywood & Boyer, 1981; Haywood, 1983), sonicated liposomes in the fluid phase would not be expected to be able to fuse. That Hsu et al. (1983) are probably measuring something other than membrane fusion is consistent with their different lipid requirements and with the fact that they demonstrated considerable lipid exchange in the cold.

Effect of Phosphatidylethanolamine (PE) upon Membrane Fusion. Whether the inclusion of PE in a liposome changes its ability to fuse with viral membranes was tested by making liposomes from 0.7 μmol of PC, varying amounts of G_{D1a}, and 0.3 μmol of PE. Comparison of Figure 2 with Figure 1 shows

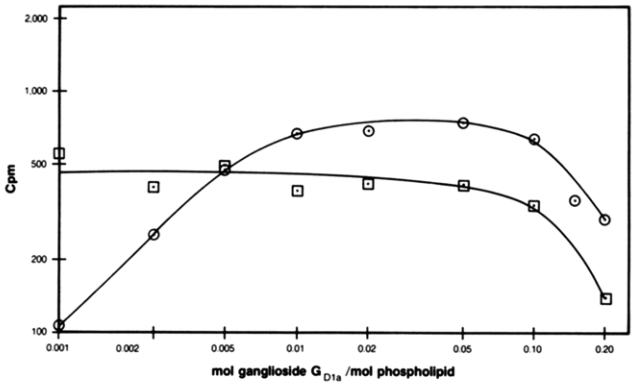

FIGURE 2: Effect of phosphatidylethanolamine and phosphatidylserine upon fusion of liposomes with virus. Liposomes were made from 0.7 μmol of PC, varying amounts of G_{D1a}, and either 0.3 μmol of PE or 0.3 μmol of PS. ^{35}S-Labeled Sendai virus (5000 cpm) was added and incubated for 2 h at 40 °C, and the amount of labeled viral protein incorporated into liposomes was counted. (○) PC, gangliosides, and PE; (□) PC, gangliosides, and PS.

Table III: Effect of Phosphatidylethanolamine Concentration upon Membrane Fusion[a]

PE (mol % of phospholipid)	viral protein (cpm) incorp into liposomes	PE (mol % of phospholipid)	viral protein (cpm) incorp into liposomes
30	1500	50	1970
40	1810	60	2090

[a] Liposomes were made from 0.05 μmol of ganglioside G_{D1a} and 1 μmol of phospholipid composed of PC and PE in different ratios. ^{35}S-Labeled virus (10000 cpm) was added and incubated for 2 h at 40 °C.

that the addition of 30 mol % PE makes little change in the amount of fusion of liposomes with Sendai virus membranes.

It was previously suggested (Haywood, 1974b, 1978) that PE at this concentration could be important for membrane fusion. Membrane fusion was being assayed by electron microscopy which showed both fusion and disassembly of the virus with distribution of the viral proteins in the liposomal membrane. While it is now clear that fusion can occur with liposomes containing only PC and G_{D1a}, it is possible that the presence of additional lipids such as PE is necessary for the viral proteins to become distributed through the liposomal membrane.

To determine if concentrations of PE higher than 30 mol % would facilitate membrane fusion, liposomes were made which contained PC, 5 mol % G_{D1a}, and varying amounts of PE. It can be seen in Table III that as the mole percent of PE increases from 30 to 60, the amount of fusion slowly increases. Sixty mole percent is higher than is usually present in mammalian membranes, but it is possible that local domains exist that contain that much PE. PE at high concentrations has been reported to facilitate Ca^{2+}-induced fusion of PS-containing liposomes (Miller & Racker, 1976; Düzgünes et al., 1981) and of PI-containing liposomes (Sundler et al., 1981) which led to the suggestion that PE might increase the amount of fusion because of the relatively poor hydration of its head group. Other properties of PE which might relate to membrane fusion are its ability to form hydrogen bonds with other phospholipid head groups (which relates to its decreased hydration) and its small head group which affects its packing properties. Egg PE can also undergo a polymorphic phase transition which has been suggested to play a primary role in membrane fusion (Cullis & de Kruijff, 1979) rather than the facilitory role observed here.

Effect of Phosphatidylserine (PS) upon Membrane Fusion. To see if the presence of PS in liposomes alters how liposomes

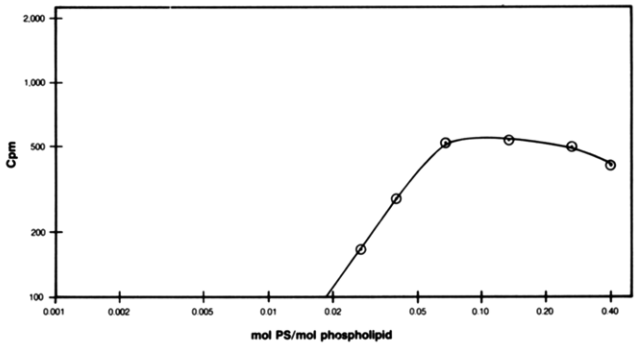

FIGURE 3: Fusion of Sendai virus membranes with liposomes containing only PC and varying amounts of PS. Liposomes were made of PC and PS in varying proportions to make a total of 1 μmol of phospholipid. ^{35}S-Labeled Sendai virus (500 cpm) was added and incubated for 2 h at 40 °C, and the amount of labeled viral protein incorporated into liposomes was counted.

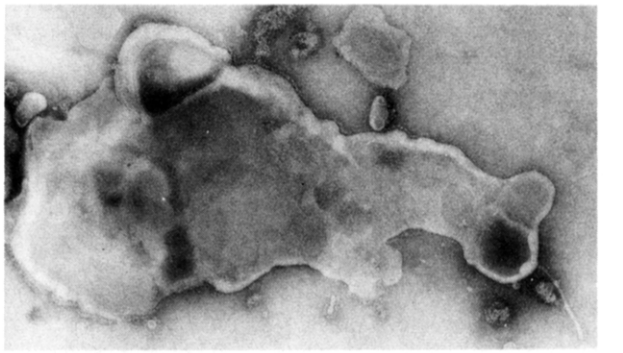

FIGURE 4: Fusion of Sendai virus with liposomes containing PC and 5 mol % PS. Liposomes were made from 0.95 μmol of PC and 0.05 μmol of PS. Unlabeled Sendai virus (67 μg of protein) was incubated with the liposomes for 2 h at 40 °C. Magnification 38700×.

fuse with Sendai virus membranes, liposomes were made that contained 0.7 μmol of PC, varying amounts of G_{D1a}, and 0.3 μmol of PS. Figure 2 shows that at high ganglioside concentrations, PS does not change the amount of fusion. In the presence of PS, however, a reduction in ganglioside concentrations does not result in a reduction in the amount of fusion.

This observation suggested that liposomes containing only PC and PS with no gangliosides might fuse with virus membranes. To test this, liposomes were made with PC and varying amounts of PS, and the amount of fusion with Sendai virus membranes was determined. As can be seen in Figure 3, fusion does occur with liposomes containing only PC and PS. As with ganglioside G_{D1a}, an increasing concentration of PS results in an increasing amount of fusion until a plateau is reached. The concentration at which fusion begins to occur is about 10 times higher with PS than with G_{D1a}, and, once this threshold is reached, the rise to the plateau is steeper with PS than with G_{D1a}.

It was previously shown that an active F protein is required for fusion of Sendai virus membranes with liposomes containing gangliosides and that the fusion assay reflects only fusion and not binding, since no fusion occurred either at 0 °C or with virus containing the inactive F_0 protein (Haywood & Boyer, 1982). Similarly, Sendai virus membrane fusion with liposomes containing PC and 5 mol % PS requires an active F protein and higher temperatures, since no fusion occurs either at 0 °C or with virus containing the inactive F_0 protein. To further confirm that fusion is occurring in the absence of gangliosides and to examine disassembly with these liposomes, Sendai virus and liposomes containing PC and 5 mol % PS were examined in an electron microscope after incubation at

40 °C for 2 h. Fusion had occurred (Figure 4), and the viral glycoprotein spikes had become distributed in the liposomal membrane. In addition, small vesicles and very narrow irregular branching structures appeared which contained viral glycoproteins. These structures are very common when viruses are incubated with liposomes containing PC and PS and have been previously reported (Haywood, 1978) to bud off liposomes that contained PC, PE, PS, sphingomyelin, cholesterol, and gangliosides. This suggests that their formation depends upon liposomal lipid composition and may relate to the interaction between viral proteins and specific lipids.

For PS-containing liposomes to fuse with each other, Ca^{2+} is required (Papahadjopoulos et al., 1979) and is thought to cause a discontinuity in lipid packing by forming a lateral phase separation (Papahadjopoulos, 1978) and/or an anhydrous Ca^{2+}–lipid complex (Portis et al., 1979). Therefore, the role of Ca^{2+} in the fusion of virus with liposomes containing PS was tested. ^{35}S-Labeled Sendai virus (5000 cpm), which had been dialyzed against HKN, and liposomes containing PC and 5 mol % PS were incubated for 2 h at 40 °C in HKN alone or in HKN with either disodium ethylenediaminetetraacetate (Na_2EDTA) or $CaCl_2$. The number of counts in viral protein incorporated into liposomes in the presence of 1 mM Na_2EDTA was 720 and in the presence of 1 mM $CaCl_2$ was 750. Therefore, the fusion is not dependent upon Ca^{2+}. When fusion occurred in HKN without either Na_2EDTA or $CaCl_2$, the number of counts incorporated was 1180. Why a greater number of counts is incorporated in the absence of both Na_2EDTA and $CaCl_2$ is not known, but it is possible that they both displace some cation which facilitates fusion with liposomes containing PC and PS. When 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was added, the amount of fusion was equal to that occurring in the presence of Na_2EDTA or Ca^{2+}. The result that Ca^{2+} is not required for fusion of virus with liposomes containing PC and PS is similar to what was previously found with liposomes containing PC, PE, cholesterol, and G_{D1a} (Haywood & Boyer, 1982). Sendai virus membrane fusion is one of the few examples of membrane fusion that does not require Ca^{2+}. A packing defect at the edge of a Ca^{2+}-induced lateral phase separation is therefore clearly not required for virus membrane fusion, although the possibility that the binding of viral protein to lipids causes a lateral phase separation cannot be eliminated.

Fusion with Liposomes Containing Only One Lipid and with Liposomes Containing Six Lipids. To see if Sendai virus membranes could fuse with liposomes containing only one lipid, ^{35}S-labeled viruses (10000 cpm) were incubated 2 h at 40 °C with liposomes containing 1 μmol of PS. Fusion did occur as shown by the incorporation of 948 cpm of viral protein into the liposome, so fusion is not dependent upon a packing defect between two different lipids or upon specific interactions between lipids in the liposome. To look at fusion with liposomes containing many lipids as occurs in biologic membranes, liposomes were made from six lipids in roughly the proportions that they appear in BHK (baby hamster kidney) cells. This was the composition used in the initial studies of fusion of Sendai virus with liposomes (Haywood, 1974a). The six lipids were 0.54 μmol of PC, 0.27 μmol of sphingomyelin, 0.12 μmol of PE, 0.06 μmol of PS, 0.05 μmol of G_{D1a}, and 0.75 μmol of cholesterol. These liposomes incorporated 1670 cpm of viral proteins and were among those that fused best with Sendai virus membranes.

Ability of Liposomes Containing PC and Lipids of Different Charge and Shape To Fuse. To see if lipids other than PS and gangliosides can confer upon liposomes the ability to fuse, liposomes were made containing PC and 5 mol % of a second lipid. When the second lipid was zwitterionic (PE or sphingomyelin) or a neutral sugar (globoside), the amount of viral protein associated with liposomes was in the same low range as with liposomes containing only PC (Table IV).

Table IV: Fusion of Sendai Virus Membranes with Liposomes Containing PC and a Second Lipid[a]

composition of liposomes	viral protein (cpm) incorp into liposomes
neutral liposomes	
PC alone	43
PC + PE	89
PC + sphingomyelin	182
PC + globoside	145
negatively charged liposomes	
PC + PI	1070
PC + PA	1170
PC + PG	1140
PC + G_{M1}	1180
PC + G_{D1a}	1310
PC + dicetyl phosphate	1630
PC + PE (pH 10)	1610

[a] Liposomes were made from 1 μmol of PC or from 0.95 μmol of PC plus 0.05 μmol of the second lipid indicated. All were made in PBS at pH 7.5 except for one set of liposomes containing PE which were made at pH 11 and, after the addition of virus in low-phosphate buffer, had a measured pH of 10. ^{35}S-Labeled Sendai virus (10000 cpm) was added and incubated for 2 h at 40 °C.

When liposomes containing 5 mol % of negatively charged lipids were incubated with virus, however, fusion clearly occurred in every case (Table IV). The amount of fusion did not depend upon the shape of the phospholipid. Thus, liposomes containing PA and PG, which have small head groups, did not fuse to any significantly greater degree than did liposomes containing PI, which has a bulky head group. The fact that the amount of fusion with liposomes containing PI is similar to that with liposomes containing PA indicates that metabolic conversion of PI to PA is not involved in liposome fusion with viral membranes. While liposomes containing phosphatidylglycerol (PG) behave like liposomes containing other negatively charged lipids, liposomes containing cardiolipin (diphosphatidylglycerol) gave results which differ from those found with other liposomes tested, and which are still under investigation. Liposomes containing the ganglioside G_{M1}, which does not act as a Sendai virus receptor (Haywood, 1975b; Markwell et al., 1981) but does carry a negative charge, also fuse. Liposomes containing dicetyl phosphate, which has an unshielded phosphate group and which is often used to provide a negative charge in liposomes, gave excellent fusion. Although PE is a neutral lipid at physiologic pH, when the pH is high enough that the amine group is no longer charged, PE carries a net negative charge due to the phosphate group. At pH 10, viruses fuse with liposomes containing PC and PE but not with liposomes containing PC alone. Thus, lipids such as ganglioside G_{D1a} which can bind virus in the cold and those that carry a negative charge but at 5 mol % do not cause binding in the cold can both give liposomes the ability to fuse. The main difference seems to be the amount of lipid required before fusion occurs. All of the lipids which give liposomes the capacity to fuse with virus are negatively charged and have an oxygen available to form hydrogen bonds. The negative charge and the available oxygen can be on either a phosphate group or a carboxyl group.

In summary, Sendai virus membrane can fuse with negatively charged liposomes made of a variety of lipid compositions, but fusion does not occur with any of the neutral liposomes tested. As discussed above, these data make it possible

to eliminate from consideration many mechanisms of liposome fusion with Sendai virus membranes that require lipids with specific properties. There are quantitative differences in the amount of fusion after a 2-h incubation at 40 °C with liposomes of different compositions. There is no clear pattern to these quantitative differences, but the ability of the liposomes to bind may be a factor. Ganglioside G_{D1a} has previously been shown to act as a receptor for Sendai virus (Haywood, 1975b; Markwell et al., 1981), and concentrations as low as 0.25 mol % impart to liposomes some capacity to fuse. When a negatively charged lipid such as PS is added to PC, it must be present in approximately a 10-fold higher concentration than G_{D1a} for fusion to occur. It has long been known that, in a medium lacking proteins, influenza virus and the paramyxovirus can nonspecifically bind to negatively charged surfaces (Valentine & Allison, 1959; Huang, 1974). While previous work indicated that Sendai virus does not bind in the cold to liposomes containing PC and 5 mol % of negatively charged lipids, such as PS (Haywood, 1974a), it seems likely that there is some binding at 40 °C. Further studies on binding are currently being undertaken. Preliminary data suggest that viruses are enveloped by liposomes containing PC and 5 mol % PS at warm temperatures as they are by ganglioside-containing liposomes (Haywood, 1975a).

Previous work (Haywood & Boyer, 1981) has shown that Sendai virus fuses with ganglioside-containing liposomes at the leading edge of a developing vacuole where the membrane is locally very curved. This leading edge can be expected to have an increased surface free energy both because of the curvature and because it is between the force due to virus binding and the membrane surface tension (Haywood, 1983). That fusion results from conditions that increase the surface free energy has also been suggested for liposome–liposome (Miller et al., 1976; Ohki, 1982) and liposome–planar (Cohen et al., 1982) membrane fusion. If fusion depends upon the activities of the viral proteins and formation of a region of increased surface free energy, then the lipid composition would not be expected to play a determining role as long as binding can occur. This is consistent with these data on the effect of liposomal composition.

Registry No. G_{D1a}, 12707-58-3; G_{M1}, 37758-47-7; Ca, 7440-70-2; cholesterol, 57-88-5.

References

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Cohen, F. S., Akabas, M. H., & Finkelstein, A. (1982) *Science (Washington, D.C.) 217*, 458–460.

Courchaine, A. J., Miller, W. H., & Stein, D. B. (1959) *Clin. Chem. (Winston-Salem, N.C.) 5*, 609–614.

Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Düzgünes, N., Wilschut, J., Fraley, R., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 642*, 182–195.

Engelman, D. M., & Rothman, J. E. (1972) *J. Biol. Chem. 247*, 3694–3697.

Evans, E. A., & Parsegian, V. A. (1983) *Ann. N.Y. Acad. Sci. 416*, 13–33.

Haywood, A. M. (1974a) *J. Mol. Biol. 83*, 427–436.

Haywood, A. M. (1974b) *J. Mol. Biol. 87*, 625–628.

Haywood, A. M. (1975a) *J. Gen. Virol. 29*, 63–68.

Haywood, A. M. (1975b) in *Negative Strand Viruses* (Barry, R. D., & Mahy, B. W. J., Eds.) Vol. 2, pp 923–928, Academic Press, London.

Haywood, A. M. (1978) *Ann. N.Y. Acad. Sci. 308*, 275–280.

Haywood, A. M. (1983) in *Liposome Letters* (Bangham, A. D., Ed.) pp 277–287, Academic Press, London.

Haywood, A. M., & Boyer, B. P. (1981) *Biochim. Biophys. Acta 646*, 31–35.

Haywood, A. M., & Boyer, B. P. (1982) *Biochemistry 21*, 6041–6046.

Hope, M. J., Bruckdorfer, K. R., Hart, C. A., & Lucy, J. A. (1977) *Biochem. J. 166*, 255–263.

Hsu, M.-C., Scheid, A., & Choppin, P. W. (1983) *Virology 126*, 361–369.

Huang, R. T. C. (1974) *Med. Microbiol. Immunol. 159*, 129–135.

Jendrasiak, G. L., & Hasty, J. H. (1974) *Biochim. Biophys. Acta 337*, 79–91.

Kundrot, C. E., Spangler, E. A., Kendall, D. A., MacDonald, R. C., & MacDonald, R. I. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1608–1612.

Lucy, J. A. (1970) *Nature (London) 227*, 814–817.

Lyles, D. S., & Landsberger, F. R. (1979) *Biochemistry 18*, 5088–5095.

Maeda, T., Asano, A., Okada, Y., & Ohnishi, S.-I. (1977) *J. Virol. 21*, 232–241.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.

Markwell, M. A. K., Svennerholm, L., & Paulson, J. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5406–5410.

Miettinen, T., & Takki-Luukkainen, I.-T. (1959) *Acta Chem. Scand. 13*, 856–858.

Miller, C., Arvan, P., Telford, J. N., & Racker, E. (1976) *J. Membr. Biol. 30*, 271–282.

Ohki, S. (1982) *Biochim. Biophys. Acta 689*, 1–11.

Papahadjopoulos, D. (1978) in *Membrane Fusion* (Poste, G., & Nicolson, G. L., Eds.) pp 765–790, North-Holland, New York.

Papahadjopoulos, D., Poste, G., & Vail, W. J. (1979) *Methods Membr. Biol. 10*, 1–121.

Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.

Sundler, R., Düzgünes, N., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 649*, 751–758.

Valentine, R. C., & Allison, A. C. (1959) *Biochim. Biophys. Acta 34*, 10–23.

Yeagle, P. L., Hutton, W. C., Huang, C., & Martin, R. B. (1977) *Biochemistry 16*, 4344–4349.

Young, J. D.-E., Young, G. P. H., Cohn, Z. A., & Lenard, J. (1983) *Virology 128*, 186–194.

Zilversmit, D. B., & Hughes, M. E. (1976) *Methods Membr. Biol. 7*, 211–259.