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Interaction of Sindbis Virus with Liposomal Model Membranes

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Radiolabeled Sindbis virus was found to bind to protein-free lipid model membranes (liposomes) derived from extracts of sheep erythrocytes. The virus interaction was dependent on initial pH, and the range of pH dependence (pH 6.0 to 6.8) was the same as that observed with virus-dependent hemagglutination. After the initial interaction, pH changes no longer influenced the virus binding to liposomes. Virus bound to liposomes prepared from a mixture of erythrocyte phospholipids, but the binding was greatly diminished when either cholesterol or phosphatidylethanolamine was omitted from the liposomal lipid mixture. It was concluded that phospholipids and cholesterol, in a bilayer configuration, may be sufficient for specific virus binding in the absence of membrane protein.

Although Sindbis virus is known to possess hemagglutinating activity (2), the erythrocyte membrane components responsible for virus attachment have not been identified. The possible role of lipids in the Sindbis virus-erythrocyte interaction has been inferred from a variety of indirect studies (8, 17, 18). Normal human serum contained chloroform-soluble inhibitors of arbovirus hemagglutinins, and lipid extracts of both avian and mammalian erythrocytes partially inhibited hemagglutination by these viruses (8, 17). Mixtures of either glycerophosphatides or long-chain fatty acids, together with cholesterol or cholesterol acetate, inhibited hemagglutination by Sindbis virus or other alphaviruses (8, 18).

The purpose of this study was to investigate the possible role of various membrane-derived lipids in the binding of Sindbis virus. These membrane lipids were incorporated into liposomal model membranes, which are protein-free particles containing concentric spheres of lipid bilayers. Liposomes have previously been used in studies on the interaction of Sendai virus with membranes (10, 11). Utilizing a simple centrifugation procedure for the separation of unattached virions, we report here that Sindbis virus binds to liposomes derived from sheep erythrocytes. The properties of this binding to liposomes closely paralleled those observed with intact erythrocytes. Detailed analysis, using mixtures of purified lipids, showed that cholesterol and phosphatidylethanolamine significantly enhanced the binding of Sindbis virus.

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MATERIALS AND METHODS

Preparation of Sindbis virus and antiserum. The preparation of intrinsically radiolabeled Sindbis virus has been described (4). Briefly, primary chicken embryo cell cultures were infected with mouse brain-propagated Sindbis virus, AR 339, at a multiplicity of infection of 1 to 10, and the infected cultures were incubated in amino acid-free medium for 4 to 6 h. A ^{3}H-labeled amino acid mixture (NET 250, New England Nuclear Corp., Boston, Mass.) was added at a concentration of 10 to 30 μCi/ml, and the supernatant fluids were harvested at 16 to 20 h postinfection. Virus-containing fluids were clarified by slow-speed centrifugation and subsequently precipitated by 60% ammonium sulfate. Virus-containing precipitates were resuspended in a minimal volume of buffer to achieve a 10- to 20-fold concentration.

Rate zonal sucrose gradient purification was accomplished on a 15 to 30% gradient centrifuged for 3 h at 25,000 rpm in a Beckman SW 25.1 rotor. Fractions were volumetrically collected from the bottom of the tube, and radioactivity was determined by counting in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.), using a scintillation cocktail of NCS (Amersham-Searle Co., Arlington Heights, Ill.), Liquifluor (New England Nuclear Corp.), and spectral-grade toluene (Matheson, Coleman, and Bell, Norwood, Ohio). A peak of radioactivity, coincident with a peak of infectivity and hemagglutinating activity, was found in the middle fractions of the gradient. Virion preparations used in these studies consisted of fractions taken from under this peak.

Fractions from different parts of the radioactive peak did exhibit some variation in binding efficiency; consequently, all of the critical experiments were performed at least once with virus material from the fraction exhibiting the highest radioactivity. Virus-containing fractions were stored at −70C, and virion structure appeared to remain intact since thawed and

diluted virion specimens resedimented in sucrose gradients without evidence of degradation.

Lipids. Sheep erythrocytes were obtained from a single animal. Erythrocyte ghosts were extracted with chloroform, methanol, and water by a modification of the method of Bligh and Dyer (1, 14). The methanol-water phase was discarded. The chloroform phase (fraction IIa) was protein free (9) but contained phospholipids, glycolipids, and cholesterol (9, 13). In some instances, these lipids were further separated by silicic acid chromatography (19). A quantity of fraction IIa, containing 100 μmol of phosphate, was applied to a column containing 10 g of silicic acid. The column was developed with 150 ml of chloroform to remove neutral lipids. The column was then developed with 450 ml of acetone-methanol (9:1) to remove the glycolipids, and the phospholipid fraction was obtained by developing the column with 500 ml of methanol.

The phospholipids were separated by thin-layer chromatography on plates precoated with Silica Gel 60 (EM Laboratories, Inc., Elmsford, N.Y.) in a solvent system of chloroform-methanol-water (65:35:4). The individual phospholipid bands were scraped and eluted on a sintered-glass funnel with chloroform-methanol (2:1). Phosphate determinations on the eluted fractions were performed by the method of Gerlach and Deuticke (6). Both the original chloroform extract (fraction IIa) and the phospholipid fraction were found to contain phosphatidylethanolamine-sphingomyelin-phosphatidylserine in approximate molar ratios of 0.45:0.45:0.1. The phospholipid fraction obtained from the silicic acid column was free of detectable glycolipids as determined by spraying thin-layer chromatography plates with 0.2% orcinol in 75% H_2SO_4, and is hereafter referred to as PL.

Sheep erythrocyte phosphatidylethanolamine was isolated by preparative thin-layer chromatography from PL. Other lipids used in this study were purchased as follows: synthetic β,γ-dipalmitoyl phosphatidylethanolamine, cholesterol, and bovine brain phosphatidylserine (Calbiochem, Los Angeles, Calif.); bovine brain sphingomyelin (Pierce Chemical Co., Rockford, Ill.); and synthetic L-α-1-stearoyl-2-oleoyl phosphatidylcholine (Applied Science Laboratories, State College, Pa.). All lipids were routinely checked for purity by thin-layer chromatography.

Preparation of the liposomes. Liposomes were prepared on a daily basis by previously described procedures (14). Chloroform solutions of the appropriate lipids were dried with a rotary evaporator. The lipid film was further dried under vacuum (<50 μm of Hg) for at least 90 min. The lipids were then dispersed in the appropriate buffer on a Vortex mixer, using a small quantity of 0.5-mm glass beads. The phospholipid composition of the liposomes was 10 mM with respect to the final aqueous dispersion.

Assay of virus attachment. The Sindbis virus was always diluted in buffer at pH 5.8. Unless otherwise indicated, 600 μliters of buffer, an appropriate quantity of liposomes prepared in the identical buffer, and radiolabeled Sindbis virus were incubated for 45 min in a total volume of approximately 800 μliters. After incubation, the entire mixture was layered over 3.8 ml of 15% (wt/vol) sucrose on a bottom 0.5-ml cushion of 70% (wt/vol) sucrose. The tubes were centrifuged at 50,000 rpm (234,000 × *g*) for 45 min in a SW 50.1 rotor (Beckman Instrument Co., Palo Alto, Calif.) at 4 C. This centrifugation was sufficient to sediment unattached virus onto the 70% sucrose cushion and caused the liposomes to float to the top of the tube (see Fig. 1). Each tube was frozen in liquid nitrogen and cut into five segments with a jeweler's hacksaw. The liquid volumes of each of the segments were measured after thawing, and 200-μliter samples were removed for radioactivity counting. Eighty-five to 90% of both the total liquid and total radioactivity originally added to the centrifuge tube was recovered. The term "percent virus bound" hereafter refers to the amount of radiolabel found with the liposomes in the top segment compared with the total counts recovered from all five segments of the tube.

RESULTS

Binding of Sindbis virus to liposomes. A typical experiment illustrating the distribution of radiolabeled virus after centrifugation in the presence and absence of liposomes is shown in Fig. 1. Virtually all of the recovered virus appeared in either the top fraction (fraction 1) or on the high-density sucrose cushion (fraction 5). Phosphate analyses performed on control tubes (not shown) containing 100 μliters of liposomal dispersion, but without virus, revealed that at least 96% of the added liposomal phosphate was routinely recovered in fraction 1. The interaction of virus with liposomes was detected by a change in the sedimentation of the virus such that it floated with the liposomes (fraction 1) instead of appearing in fraction 5.

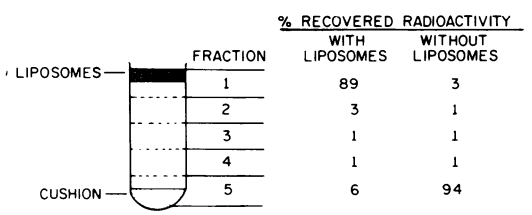
In separate experiments, pretreatment of radiolabeled Sindbis virus with antiviral neutralizing antibody completely inhibited virus attachment to sheep erythrocyte liposomes, and all radioactivity quantitatively sedimented to the bottom of the centrifuge tube (fraction 5). This result is consistent with the interpretation that all of the radiolabeled material corresponded to virus and was recognized as such by specific antibody.

Binding of Sindbis virus to liposomes derived from erythrocytes. Sindbis virus hemagglutination exhibits a strict pH dependence, with maximal agglutination below pH 6.0 and minimal reaction above pH 6.8 (2). Although goose erythrocytes have been routinely used in the past for alphavirus hemagglutination, sheep erythrocytes agglutinated as well as avian cells (Fig. 2). A maximal hemagglutination titer of 1:640 and a pH optimum of 6.0 were observed with each of the cell types. For these reasons, and because the lipid composition of sheep erythrocytes has been more thoroughly studied,

subsequent experiments utilized liposomes prepared from sheep rather than avian erythrocytes.

Liposomes from sheep erythrocytes exhibited a pH dependence for virus attachment similar to that observed for hemagglutination. Thus, maximal binding occurred at pH 6.0 and minimal binding occurred above pH 6.8 (Fig. 3). It should be noted that the pH dependence of virus binding to liposomes was not absolute, and a low level of binding (at least 18%) was still observed above pH 6.8. In contrast to erythrocytes, which cannot be easily tested in a hemagglutination assay below pH 5.8, liposome binding was measured over a more acid range, and near-maximal binding was consistently observed between pH 3.3 and 6.9. Control experiments demonstrated that, with respect to sedimentation characteristics, the virus itself was not altered by low pH and was recovered in fraction 5 of a gradient lacking liposomes similar to that shown in Fig. 1.

The pH dependence of virus binding suggested the possibility that the interaction might be due to electrostatic bonds. However, the influence of pH appeared to be important only during the initial association of the virus with the liposomes (Table 1). When the pH was raised to pH 6.7 after the initial interaction, the virus-liposome complex remained stable and



FRACTION	% RECOVERED RADIOACTIVITY WITH LIPOSOMES	% RECOVERED RADIOACTIVITY WITHOUT LIPOSOMES
1	89	3
2	3	1
3	1	1
4	1	1
5	6	94

FIG. 1. *Centrifugation characteristics of Sindbis virus and liposomes. Each tube contained 14,000 counts/min of radiolabeled virus, either with or without liposomes prepared from sheep erythrocyte extracts. 100 μliters of liposomes was incubated with virus at 37 C, using borate-phosphate-saline buffer (2) at pH 5.8. The values shown are the means of either five experiments (with liposomes) or four experiments (without liposomes).*

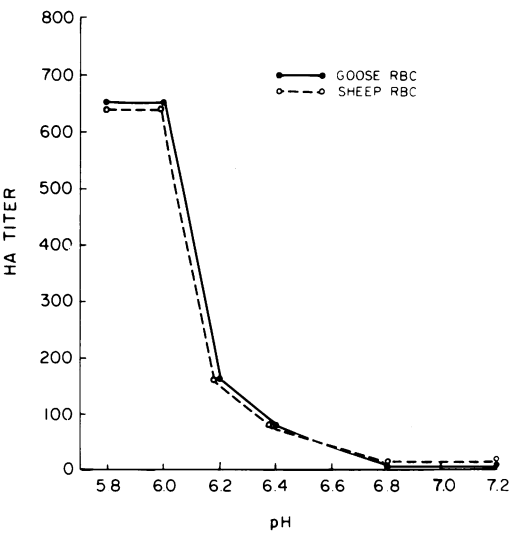


FIG. 2. *Sindbis virus hemagglutination of goose and sheep erythrocytes at 37 C. Serial 1:2 dilutions of virus were made in microtiter plates and erythrocytes were added in pH-adjusting diluent (2). "HA titer" is defined as the reciprocal of the highest dilution exhibiting positive agglutination. Each point represents the mean of duplicate determinations.*

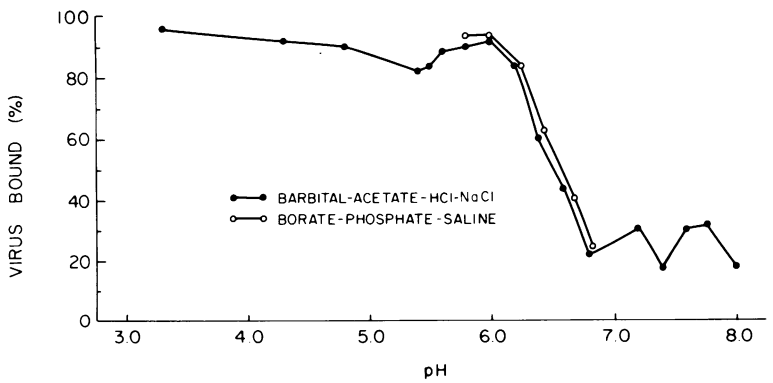


FIG. 3. *pH dependence of Sindbis virus attachment to liposomes. Each point represents a separate liposome preparation. The isotonic barbital-acetate-HCl-NaCl buffers were prepared as follows: 10 ml of 0.16 M sodium barbital-0.143 M sodium acetate was mixed with 1.0 N HCl to achieve the desired pH. The solution was made isotonic by adding appropriate quantities of 1.54 M NaCl and water to a final volume of 50 ml. 100 μliters of sheep erythrocyte liposomes, swollen in the indicated buffer at the appropriate pH, was incubated in the same buffer with radiolabeled Sindbis virus at 37 C.*

TABLE 1. *pH stability of the virus-liposome complex*[a]

pH of initial mixture	Final pH	% Virus bound
5.8	5.8	94
5.8	6.7	92

[a] 100 μliters of sheep erythrocyte liposomes, swollen in isotonic phosphate-buffered saline, pH 5.8, was incubated with radiolabeled virus for 25 min at 37 C. Where indicated, the pH was then raised to pH 6.7 by adding an appropriate volume of buffer of pH 7.4. As a control, an identical volume of pH 5.8 buffer was added to the other tube, and incubation was continued for 20 min. Each value represents an average of two experiments.

did not dissociate. These results suggest that, although electrostatic forces may be important, other bonds may also play a role in the phenomenon.

Binding to liposomes prepared from pure lipids. From the above experiments, it was concluded that Sindbis virus can bind to protein-free lipid substrate. The liposomes used above consisted predominently of a mixture of various phospholipids, cholesterol, and glycolipids (9, 14). In separate experiments, with liposomes containing only phospholipids and cholesterol, it was found that reconstitution of the liposomes with sheep erythrocyte glycolipids did not have any influence on virus binding. Furthermore, removal of all the glycolipids from mixtures of sheep erythrocyte lipids also did not have an adverse effect on binding. The following experiments were therefore devised to further clarify the relative roles of the major individual lipid constituents, other than glycolipids, in the attachment of Sindbis virus.

Radiolabeled Sindbis virus was incubated with liposomes prepared from either PL and cholesterol or PL alone (Fig. 4). The amount of bound virus is illustrated as a function of the volume of liposomes. Liposomes containing cholesterol bound virus very readily and reached a maximal value at about 1.25 μliters of liposomes. In the absence of cholesterol, the binding of virus was significantly decreased. Under these conditions, 20 μliters of liposomes gave a mean binding of 33%. The values shown were significant at every liposome concentration (P always < 0.005).

The influence of cholesterol concentration is illustrated in Fig. 5. Virus binding to liposomes gave a sigmoid curve with increasing cholesterol. The highest binding was observed at a ratio (0.75) similar to that found in erythrocyte membranes (3).

Experiments were subsequently conducted to determine the relative influence of phospho-

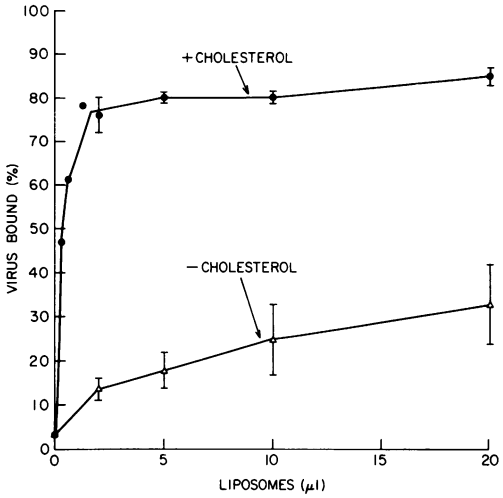


FIG. 4. *Virus binding to liposomes with and without cholesterol. Sindbis virus was incubated at 23 C with different quantities of lipsomes containing 2 μmol of mixed sheep erythrocyte phospholipids with and without 1.5 μmol of cholesterol and dispersed in 200 μliters of isotonic phosphate-buffered saline, pH 5.8. The curve involving liposomes containing cholesterol (+cholesterol) is an average of three experiments with samples from three different virus preparations. The curve of liposomes lacking cholesterol (−cholesterol) is an average of five experiments using three different virus preparations. The brackets represent 1 standard error.*

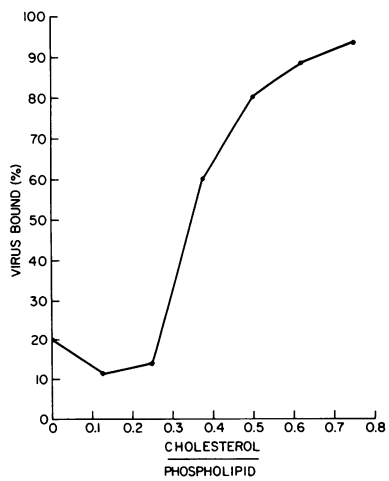


FIG. 5. *Effect of cholesterol concentration on virus binding. A single peak fraction of radiolabeled Sindbis virus was incubated with 20-μliter samples of liposomes at 23 C. Each point represents the liposomes containing the different molar ratios of cholesterol:phospholipid, as indicated. Each value represents the mean of three individual assay tubes.*

lipids on virus binding (Fig. 6). Liposomes containing sphingomyelin, phosphatidylserine, cholesterol, and either containing or lacking phosphatidylethanolamine were incubated with Sindbis virus. Marked enhancement was observed in the presence of phosphatidylethanolamine. Although some differences in virus binding did occur with different liposomes lacking phosphatidylethanolamine, the absolute values of binding were always relatively low.

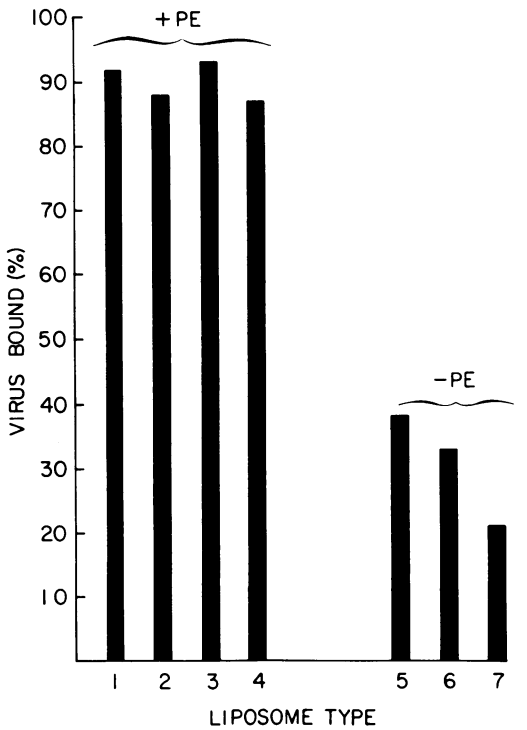


FIG. 6. *Virus binding to liposomes containing mixtures of different phospholipids. Sindbis virus was incubated at 23 C with 20 μliters of liposomes containing one of the following molar mixtures: (mixture 1) PL-cholesterol (2:1.5), (mixture 2) sheep erythrocyte phosphatidylethanolamine-sphingomyelin-phosphatidylserine-cholesterol (1:1:0.22:1.5); (mixture 3) sheep erythrocyte phosphatidylethanolamine-phosphatidylserine-cholesterol (2:0.22:1.5); (mixture 4) sheep erythrocyte phosphatidylethanolamine-sphingomyelin-cholesterol (1:1:1.5); (mixture 5) sphingomyelin-phosphatidylserine-cholesterol (2:0.22:1.5); (mixture 6) sphingomyelin-(1-stearoyl-2-oleoyl) phosphatidylcholine-phosphatidylserine-cholesterol (1:1:0.22:1.5); (mixture 7) (1-stearoyl-2-oleoyl) phosphatidyl-choline-phosphatidylserine-cholesterol (2:0.22:1.5). Each bar represents the mean virus binding to 20-μliter liposome samples observed in three assay tubes. The same peak fraction of radiolabeled virus was used with mixtures 1 through 3 and 5 through 7. Two other peak fractions of virus were used in the experiments with mixture 4.*

The influence of phosphatidylethanolamine concentration is illustrated in Fig. 7. As with cholesterol, there was a striking increase in the binding when virus was incubated with liposomes containing phosphatidylethanolamine compared with those lacking phosphatidylethanolamine. The curves illustrated in Fig. 7 are significantly different (P always < 0.001). These experiments demonstrate that phosphatidylethanolamine and cholesterol may both be important in the binding of Sindbis virus.

DISCUSSION

The erythrocyte receptor for Sindbis virus hemagglutinin has not been previously defined. Using a simple centrifugation technique, we have demonstrated that the purified Sindbis virion can bind to protein-free lipid model membranes (liposomes). Hemagglutination of erythrocytes and binding of the virus to liposomes were both similar in that the ranges of pH dependence were identical. Analysis of individual lipid requirements demonstrated that lipo-

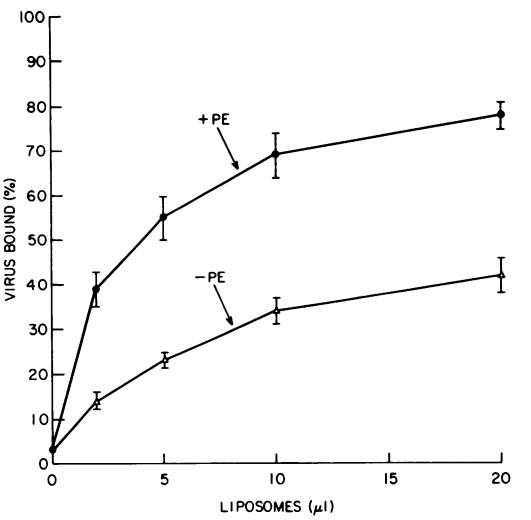


FIG. 7. *Influence of phosphatidylethanolamine on virus binding. Sindbis virus was incubated at 23 C with different quantities of liposomes containing one of the following molar mixtures: dipalmitoylphosphatidylethanolamine-sphingomyelin-phosphatidylserine-cholesterol (1:1:0.2:1.5) (+PE) or sphingomyelin-phosphatidylserine-cholesterol (2:0.22:1.5) (−PE). The curve of liposomes containing phosphatidylethanolamine is an average of five experiments with samples from three different virus preparations. The curve of liposomes lacking phosphatidylethanolamine is an average of five experiments with samples from four different virus preparations, including the three preparations used in the liposome experiments containing phosphatidylethanolamine. Each bracket represents 1 standard error.*

somes prepared from mixtures of phospholipids alone were sufficient to bind Sindbis virus. Marked enhancement was observed when cholesterol was also present in the liposomes, and binding was greatly diminished when phosphatidylethanolamine was omitted from the mixture of phospholipids and cholesterol. The ratios of phospholipids and cholesterol that gave optimal binding were the same as those found in the intact sheep erythrocyte.

Liposomes have been previously used as a model membrane to investigate the interaction of a myxovirus (Sendai) with its ganglioside receptor (10, 11). It is a matter of some interest that Sendai virus did not fuse with liposomes containing gangliosides when sphingomyelin and phosphatidylethanolamine were omitted (11). In our studies, we did not observe any requirement for ganglioside or other erythrocyte glycolipids. With Sindbis virus, we found that phosphatidylethanolamine greatly enhanced binding, but sphingomyelin was not found to have any influence. In the case of both Sendai and Sindbis viruses, therefore, phospholipid composition may have a profound, although possibly different, influence on virus binding to certain membranes.

Cholesterol has been previously shown to have both receptor properties for polyene antibiotics (12) and to interact with certain phospholipids causing reorientation of the bilayer molecules (5). Although we have observed that cholesterol is important in the binding of Sindbis virus, the mechanism of the influence of cholesterol is not yet clear. In preliminary studies we have found, however, that various other sterols (e.g., coprostanol, cholestanol, cholestanone) and cetyl alcohol can be substituted for cholesterol.

It may be premature to transpose this information obtained from erythrocyte membranes and liposomes toward speculation concerning the specific site of virus attachment to susceptible cells and the mechanism of infection. It is true, however, that both cholesterol and phospholipids are ubiquitously distributed in mammalian cell membranes. The relative geometric distribution of phospholipids and cholesterol in membranes of cells susceptible to viral multiplication has not yet been determined. In the intact erythrocyte membrane, however, the various phospholipids and cholesterol are spatially arranged in an asymmetrical distribution (7, 15, 16, 20, 21). It has been suggested that phosphatidylethanolamine and phosphatidylserine are situated predominantly on the internal surface of the erythrocyte membrane (15, 20). It is possible that different geometric arrangements of phospholipids and cholesterol might contribute to virus-receptor specificity.

From the present studies it can be concluded that liposomes may be useful as a model membrane to determine the binding characteristics of virus to lipids. It is clear from these experiments that neither cell membrane proteins nor glycolipids are required for at least the initial step of Sindbis virus attachment to lipid membranes, but that phospholipid and cholesterol may be very important.

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