LIPID VESICLE-CELL INTERACTIONS

I. Hemagglutination and Hemolysis

FRANCIS J. MARTIN and ROBERT C. MACDONALD

From the Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201. Dr. Martin's present address is the Department of Chemistry, University of California, Berkeley, California 94720.

ABSTRACT

The interaction of lipid vesicles (liposomes) of several different compositions with erythrocytes has been investigated. Lecithin liposomes, rendered positively charged with stearylamine, exhibit potent hemagglutination activity in media containing low concentrations of electrolytes. The hemagglutination titer is found to be a linear function of the zeta potential of the lipid vesicles. Hemagglutination is reduced when the surface potential of the cells is made more positive by pH adjustment or enzyme treatment. Similarly, hemagglutination is reduced by increasing concentrations of electrolytes. Hemagglutination is examined theoretically and is shown to be consistent with vesicle-cell interactions that are due to only electrostatic forces. Vesicles containing lysolecithin in addition to lecithin and stearylamine cause lysis of erythrocytes, provided the lipids of the vesicles are above the crystal-liquid crystal phase transition temperature. In addition, hemolysis requires close juxtaposition of the vesicle to the cell membrane; vesicles precoated with antibodies exhibit severely diminished hemolytic activities, only a small fraction of which can be attributed to a reduction in hemagglutination titer. Evidence is presented indicating that a single vesicle is sufficient to lyse one cell. With regard to hemagglutination and hemolysis, lipid vesicles of simple composition mimic paramyxoviruses such as Sendai virus.

Infection of cells by paramyxoviruses involves attachment of the virus membrane to the cell membrane and subsequent fusion of the two membranes (10). These membrane-associated events of viral infection may be taken as a paradigm of a large variety of cellular processes that follow a general adhesion-fusion sequence, such as synaptic transmission, fertilization, and many secretory processes. For studying such phenomena, viruses have the advantage that the special properties of their membranes are manifest in several different ways, among which are agglutination of cells, lysis of erythrocytes, fusion of cultured cells, and incorporation of antigen into host cell membranes. We have used the membrane-associated activities of the paramyxovirus Sendai as guides in developing lipid bilayer vesicles as a model system for adhesion and fusion processes of membranes. This paper describes the properties of lipid vesicles that mimic the hemagglutination (HA) and hemolytic behavior of Sendai virus. The two following papers in this series deal with the use of lipid vesicles to effect cell fusion and antigen transfer. In addition to providing basic information on membrane-

membrane interactions, lipid vesicles that behave like paramyxovirus membranes, because they may be used to introduce proteins, nucleic acids, and lipids into intact cells, offer the cell biologist a new method for studying cell function.

Lipid vesicles or liposomes of compositions other than those we have used have been investigated by others for activities that are the same or similar to those we have studied (5, 7, 8, 11, 12, 15, 16, 23–27, 31, 32). In general, the objectives of those investigations were limited to accomplishing one or two operations with liposomes, and, indeed, in most instances significant progress was reported. The major difference between our work and that of others is that our objective has been to mimic, with vesicles of the same composition, the entire range of membrane-associated activities of a particular natural membrane. A description of the initial research in this direction has appeared elsewhere (18).

MATERIALS AND METHODS

Cells

Erythrocytes from sheep or human whole blood drawn into Alsever's solution (equal volumes) were washed three times with phosphate-buffered saline (PBS). The erythrocytes were then suspended in 0.3 M sucrose containing 0.05 M phosphate buffer (pH 7.2) at a concentration of 10% (vol/vol) for hemolysis experiments and 0.5% (vol/vol) for HA assays. Hemolysis experiments and microscopy were carried out using fresh cells. Neuraminidase treatment involved incubating a 10% suspension of cells in normal saline with 50 μg/ml of the *Clostridium perfringens* enzyme (Sigma Chemical Co., St. Louis, Mo.) for between 5 min and 1 h at 37°C. The treated cells were then washed in PBS and fixed according to the procedure of Tenforde (30). Cells so fixed exhibit the same electrophoretic behavior as untreated cells over the pH range from at least 4 to 10.

Lipids

Diisostearoylphosphatidylcholine (L) and dimyristoylphosphatidylcholine (DML) were prepared by reaction of the appropriate anhydride with glycerophosphorylcholine in the presence of the triethylamine salt of the corresponding fatty acid (14). Lysophosphatidylcholine (LL) was prepared from L by hydrolysis with snake venom phospholipase. All the lipids were purified by silica gel chromatography and were free of contaminants by normal criteria of thin-layer analysis. The surfactants used in these studies were purchased from either K and K Laboratories, Inc., Plainview, N. Y. or Lachat Chemicals, Inc., Mequon, Wis. Stearylamine (SA) purity was 99.5%. Stock solutions of lipids were made in chloroform-methanol (4:1) at a concentration of 10 mg lipid/ml solvent.

Zeta Potential Measurements

The electrophoretic mobility of liposomes was determined in a microelectrophoresis apparatus of the type described by Bangham et al.(1). The potential (ζ) at the plane of shear was calculated from the electrophoretic mobility (μ) according to:

$$\zeta = \frac{4\pi\eta\mu}{D},$$

where D is the dielectric constant and η is the viscosity of the medium. Up to about 60 mV, zeta and surface potentials are essentially identical, but at higher potentials ζ progressively underestimates the surface potential (4).

Preparation of Liposomes

Positively charged liposomes were prepared by dissolving 5 mg of the desired mixture of SA, LL, and L in 5 ml of chloroform containing a few percent methanol. The solution was placed in a 50-ml round bottom flask, and the solvent was removed under reduced pressure on a rotary evaporator at a water bath temperature of 40°C. The flask was flushed with nitrogen, and 1 ml of 0.3 M sucrose plus 0.05 M phosphate buffer (pH 7.2) was added. The lipid film covering the glass was dispersed by shaking. An aliquot of the dispersion was diluted, and zeta potentials were determined as described above. The liposomes were then subjected to ultrasonic irradiation (Biosonik IV, Bronwill Scientific, Rochester, N. Y., 0.5 maximum output for 1 min at room temperature). The resulting dispersion was used for HA experiments. For hemolysis experiments, it was diluted to 2.5 mg/ml with an equal volume of buffered sucrose. Negatively charged liposomes were prepared in the same manner except that dicetylphosphate (DCP) was used in place of SA. Uncharged vesicles contained neither SA nor DCP.

A variety of molecules were tested for their suitability for the preparation of charged liposomes. Among the long chain cations tested in addition to SA were eicosanyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, hexadecylamine, dioctylamine, and oleylamine. Based upon zeta potential measurements and calculations therefrom using diffuse double layer theory (6), only stearylamine and oleylamine were stoichiometrically incorporated into liposomes in amounts up to at least 5% (wt/wt). The zeta potential of oleylamine liposomes diminished over the course of several hours, presumably as a result of autoxidation, whereas that of stearylamine liposomes remained constant for at least 12 h. Stearylamine was

hence the molecule of choice. By these same criteria, dicetylphosphate was found suitable for preparation of negative liposomes. Liposome composition is given in weight percent.

Hemagglutination Measurements

The HA titer of liposome dispersions was determined by a modified Salk pattern method (29). Serial twofold dilutions of liposome dispersions (0.025 ml) were made in 0.3 M sucrose (buffered with 50 mM phosphate, pH 7.4). An equal volume of a 0.5% suspension of washed sheep erythrocytes was added to each tube. The lowest concentration of liposomes which produced agglutination, i.e., a positive pattern on the tube bottom after the cells had settled for 2 h at 24°C, was scored as 1 HA U/ml. Triplicate assays gave identical titers.

Hemolysis Measurements

For hemolysis assays, 1 ml of the liposome dispersion to be tested was added to an equal volume of a 10% sheep erythrocyte suspension, and the cells were allowed to agglutinate in the cold for 15 min. The tubes were then transferred to a 37°C water bath. After a 30-min incubation, the cells were pelleted. The supernate was decanted and mixed with an equal volume of a solution of sodium dodecyl sulfate (SDS, final detergent concentration was 1%). The absorbance of this solution was determined at 540 nm against a blank containing 0.3 M sucrose and 1% SDS. 100% hemolysis was taken to be the absorbance of the solution obtained by adding the SDS solution directly to the cell suspension.

Microscopy

Phase microscopy and electron microscopy of negatively stained liposome-treated erythrocytes were carried out essentially as described previously (18). In brief, fresh human cells were lysed at the air-water interface and a glass rod which had been immersed in the desired liposome dispersion was then touched to this surface. For phase microscopy, the ghosts were picked up on glass coverslips and allowed to dry before examination under the microscope. For electron microscopy, the ghosts were picked up on parlodion-coated grids, stained with phosphotungstate, and then examined. So that liposomes could be easily distinguished from other membranous material under the electron microscope, they were specifically tagged with a ferritin-conjugated antibody. In that case, liposomes contained in addition to the usual lipids, a lipid antigen (the *N*-dinitrophenyl-6-aminocaproyl derivative of phosphatidylethanolamine, DNP-Cap-PE). Treatment of such liposomes with rabbit anti-dinitrophenyl serum (anti-DNP) followed by ferritin-conjugated goat anti-rabbit IgG produced specific labeling of liposome or liposome-derived membranes with ferritin. The details of this procedure are given in the third paper of this series (21).

RESULTS

Effect of Liposome and Erythrocyte Surface Charge on HA

Liposomes containing only L and suspended in 0.3 M sucrose containing 0.05 M tricine buffer (pH 7.5) exhibit no net surface charge, i.e., are stationary in an electric field. When DCP is included in such liposomes, they acquire a negative zeta potential up to −50 mV at 5% DCP. Uncharged and negatively charged vesicles exhibit no HA activity as measured by the Salk pattern assay. Liposomes containing varying amounts of the long chain cation, SA, on the other hand, agglutinate erythrocytes. Fig. 1 (filled circles) shows the relationship between HA titer and positive surface potential for liposomes containing from 0.2 to 5% SA. From 22 mV upwards, there is a linear relation between the log HA titer and the zeta potentials of the liposomes. (HA titers less than 1 are not measurable under assay conditions.) This relation breaks down at high potentials where zeta and surface potentials diverge appreciably. Positively charged liposomes also agglutinate a number of other cell types including EATC, HeLa, KB, rabbit kidney and VERO cells.

Given the apparent electrostatic nature of interaction of lipid vesicles and cells, the surface potential of the cells would be expected to play a crucial role in the HA reaction. To test this hypothesis, we examined the effect of varying the erythrocyte zeta potential on agglutination by liposomes possessing a constant positive zeta potential. In these experiments, erythrocytes, bearing surface potentials ranging from −45 to −13 mV, were prepared by treating the cells with neuraminidase for varying lengths of time. As shown in Fig. 1 (open circles), there is a linear relationship between the log HA titer and the erythrocyte potential. The liposomes contained 5% SA (zeta potential = 50 mV).

The dependence of hemagglutination on both the sign and magnitude of the erythrocyte surface charge was confirmed by a second method utilizing the dependence of the erythrocyte surface potential upon pH. As shown in Fig. 2 (triangles), the zeta potential of glutaraldehyde-fixed

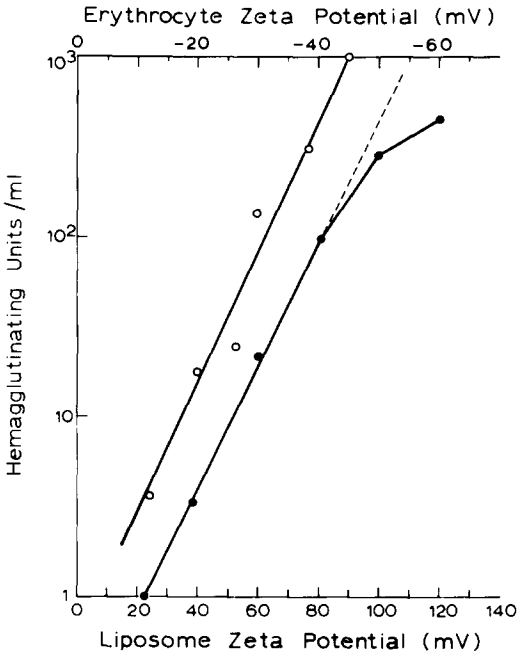

FIGURE 1 Hemagglutination titer as a function of zeta potential of liposomes or of erythrocytes. Vesicles were prepared by combining various proportions of L with SA (0.2–5% by weight of total lipid) in chloroform-methanol, removing the solvent under vacuum and dispersing the resulting lipid films in 0.3 M sucrose containing 10^{-4} M EDTA and 0.05 M phosphate buffer (pH 7.4) by mild ultrasonic irradiation. Erythrocytes with zeta potentials varying from −13 to −45 mV were prepared by treating erythrocytes with neuraminidase for various lengths of time. These cells, after washing in PBS, were resuspended in sucrose-EDTA-phosphate buffer. HA titer (Salk pattern method) was determined for untreated erythrocytes as a function of liposome zeta potential (filled circles) and for neuraminidase-treated erythrocytes as a function of their zeta potential using liposomes of constant composition, by weight, 95% L and 5% SA (open circles).

erythrocytes increases from −40 mV to +40 mV as the pH is lowered from 9 to 1. The extent of agglutination by positive liposomes (Fig. 2, circles) falls with increasing erythrocyte potential, becoming negligible as the latter reverses charge around pH 2.5.

Effect of High Concentrations of Liposomes on HA

The Salk agglutination test yields a linear and unambiguous measure of positively charged liposomes up to a concentration of about 10 mg/ml for liposomes containing 5% SA. At this concentration, buttons of agglutinated cells do not form in the first well. At 20 mg/ml, they do not form in the first two wells, and further doubling of liposome concentration increases by one the number of wells in which the erythrocytes do not agglutinate. (Buttons do, of course, form again when the liposome concentration is reduced to less than 10 mg/ml, so that the end point remains a direct measure of the liposome concentration in the first well.) The reason for the lack of agglutination at high concentrations is that the cells become coated with positive liposomes and electrostatic repulsion prevents their adhesion. This is clearly revealed by electrophoresis of erythrocytes in the presence of increasing numbers of liposomes. As shown in Fig. 3 (left axis, open circles), the zeta potential of the cells reverses sign from negative to positive as the liposome concentration is increased from 5 to 10 mg/ml. The maximum value of the positive potential (43 mV) is nearly as high as that of the liposomes before sonication (50 mV), indicating that the cells are nearly covered with liposomes. Charge reversal is also paralleled by a sharp drop in the

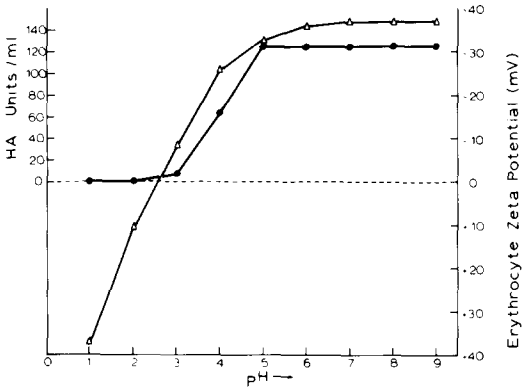

FIGURE 2 Erythrocyte zeta potential (right axis) and HA titer (left axis) as a function of pH. Washed sheep erythrocytes were fixed in PBS containing 2% glutaraldehyde for 2 h at room temperature. The cells were washed repeatedly with PBS to remove excess glutaraldehyde. The cells were resuspended in 0.3 M sucrose containing 10^{-4} M EDTA, and the pH was adjusted with either HCl or NaOH. The extent of erythrocyte agglutination by liposomes containing 5% SA was determined at pH's ranging from 1 to 9 U. In the pH range 2–9, the zeta potential of the liposomes remains essentially unchanged (about 50 mV). At pH's below 2, the vesicle surface charge becomes slightly more positive.

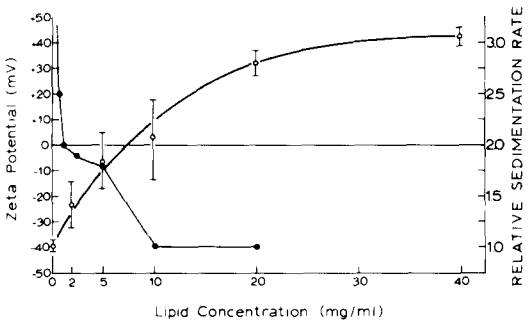

FIGURE 3 Zeta potential and sedimentation rate of erythrocytes as a function of concentration of positive liposomes. For electrophoresis (open circles), erythrocytes were suspended in 0.3 M sucrose containing 10^{-4} M EDTA, 0.05 M phosphate buffer (pH 7.4), and various concentrations of liposomes. Bars represent standard deviations of the potentials of 10 cells. For sedimentation rate (filled circles) determinations, 0.5 ml of a 0.5% suspension of washed erythrocytes was mixed with an equal volume of liposome dispersion in 0.3 M sucrose buffered (0.05 M phosphate) at pH 7.4, and the rate of cell settling at 1 *g* was measured. Sedimentation rates relative to that of untreated erythrocytes are given on the right axis. Liposomes for both sedimentation and electrophoresis experiments were 5% SA and 95% L.

rate at which treated erythrocytes settle out of suspension. The filled circles in Fig. 3 represent sedimentation rates relative to untreated cells (right axis). This rate drops to unity as the cells become positively charged, indicating that the cells become fully dispersed. The maximum sedimentation rate (maximum aggregate size) was at 4 HA U (0.04 mg/ml), a concentration too low to be included in the figure.

Effect of Monovalent Salts on HA

Because of the double layer screening effect (6), increasing concentrations of electrolytes reduce the overall surface potential of membranes. With a view to using this effect to control liposome-cell interactions, we examined the effect of monovalent salt concentration on HA. Fig. 4 shows that as the difference in absolute value of erythrocyte and liposome zeta potentials decreases from 60 to 40 mV by the addition of increasing amounts of NaCl, the HA titer drops 1,000-fold. The increase in electrolyte concentration necessary to effect this large reduction in HA titer is, as shown in the *inset*, quite small.

Effect of Lipid Composition on Hemolysis

Lysolecithin (LL) is a potent lytic agent in aqueous solution. Since, according to the data presented above, the interactions of liposomes with cell membranes can be controlled by SA content, electrolyte concentration, and pH, it appeared likely that if LL were incorporated into liposomes, their lytic activity would be subject to the same controls and would, in addition, be directed to the specific area of contact between liposome and cell membrane. As shown in Fig. 5, the hemolytic activity of L-LL-SA liposomes is indeed a function, specifically, a linear function, of both LL content and zeta potential. Adhesion and lysis are clearly independent events, for liposomes without LL are not lytic even though they may possess a very large positive charge. Liposomes that are uncharged or negatively charged (with up to 7.5% dicetylphosphate, not shown) are hemolytically inactive in spite of large lysolecithin contents.

The time-course of hemolysis has also been investigated, and it was found that, at 1.25 mg/ml, L-LL-SA liposomes produced lysis of 100% of the sheep erythrocytes in a 10% suspension within 2 h.

A series of control experiments was carried out

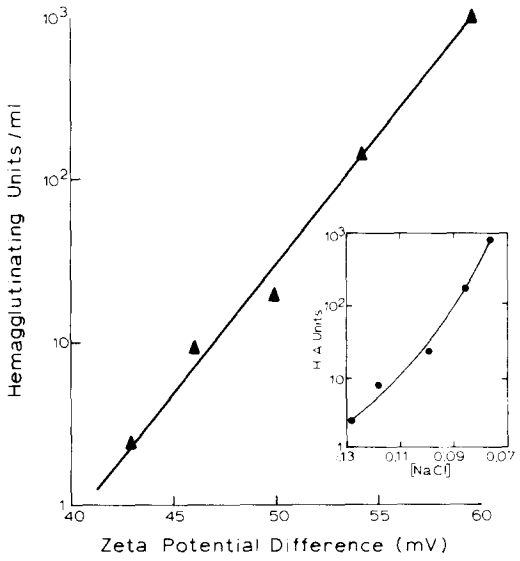

FIGURE 4 HA titer plotted vs. the absolute value of the difference between erythrocyte and liposome zeta potentials. HA titer for liposomes containing 5% SA was determined in the presence of varying concentrations of NaCl (*inset*). The zeta potentials of both liposomes and cells was calculated for each NaCl concentration. The absolute value of the difference between erythrocyte and liposome zeta potentials at each NaCl concentration is plotted against the HA titer of the liposomes at the corresponding NaCl concentration.

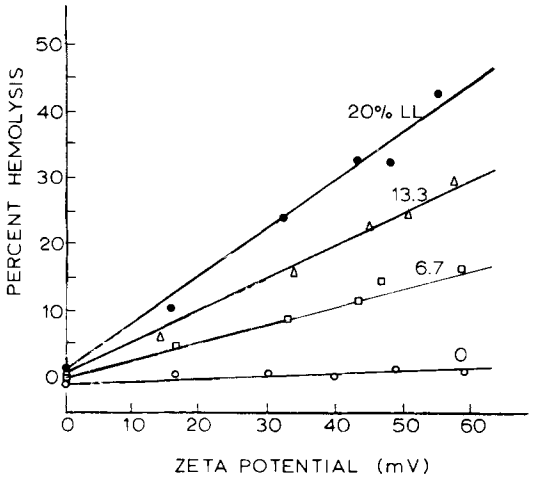

FIGURE 5 Percent hemolysis as a function of liposome zeta potential and percent LL contained in the liposome membrane. Sheep erythrocytes were treated with liposomes containing either 0, 6.7, 13.3 or 20% LL, and bearing surface potentials ranging from 0 to about 60 mV. Hemoglobin released from the cells during a 30-min incubation at 37°C in the presence of the liposomes was used as a measure of hemolysis (see text).

to assure that agglutination and hemolytic effects exhibited by lipid dispersions were not due to free or micellar SA or LL. Liposomes consisting by weight of L:SA = 10:1 and L:LL:SA = 75:15:10 were prepared. These liposomes were sonicated for 5 min (five times longer than normal to maximize the amount of lipid that became exposed to the aqueous phase [2]) and centrifuged at 60,000 rpm for 2 h. In both cases, over 95% of the lipid was sedimented. (Micelles of SA and LL do not sediment under these conditions.) Pellets were resuspended in buffer to the original volume and tested along with supernates for activity. The pellet contained over 95% of the HA activity in both cases, and 95% of the hemolytic activity in the case of the ternary mixture. Furthermore, by thin-layer chromatography, the proportions of the components exhibited no obvious differences between supernate and pellet. Finally, as noted in Materials and Methods, the calculated surface charge density of SA-L liposomes indicates stoichiometric incorporation of SA.

Effect of Liposome Concentration on Hemolysis

The relationship between percent hemolysis and liposome concentration (as measured by HA titer) is shown in Fig. 6. A linear correlation exists between log of the number of nonhemolyzed cells and the concentration of liposomes. This correlation conforms with the Poisson distribution for $n = 0$ and suggests that a single liposome is sufficient to lyse an erythrocyte. This is the same test that indicates that a single Sendai virus particle is sufficient to lyse an erythrocyte (22).

Dependence of Hemolysis on Liposome Membrane Fluidity

Lipids in natural and artificial membranes often undergo a thermal phase change from a crystalline to a liquid-crystalline (two-dimensional fluid) state at a temperature (T_c) characteristic of the lipid composing the membrane. Since recent experimental evidence suggests that the fluidity of membranes is an important factor in several intermembrane phenomena (5, 11, 24, 26), it was of interest to determine the effectiveness of hemolysis by liposomes above and below their phase transition temperature.

Phase transition temperatures for liposomes used in hemolysis experiments were determined by turbidity measurements as described previously (33). Liposomes composed of DML alone

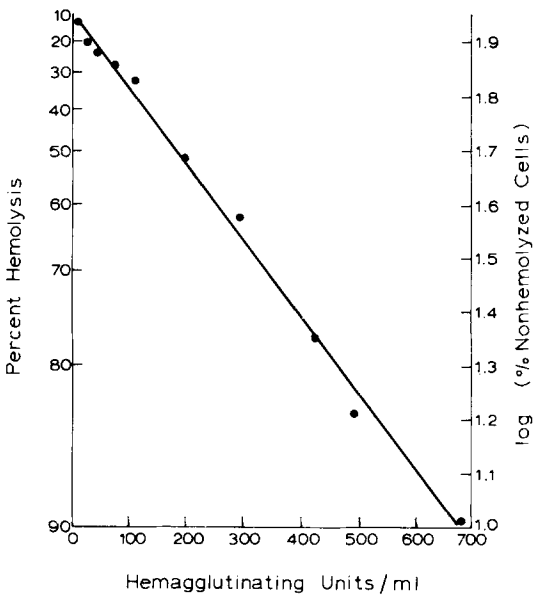

FIGURE 6 Percent hemolysis (left axis) and log (% nonhemolyzed cells) (right axis) plotted vs. liposome concentration (as measured by HA titer). The extent of hemolysis by liposomes composed of 20% LL, 5% SA, and 75% L was determined at various liposome concentrations as described in Materials and Methods.

exhibit a sharp phase transition at 24°C but, as would be expected from earlier results, were devoid of hemolytic activity above and below this temperature. Liposomes composed of DML plus LL and SA exhibit a broader but distinct transition, beginning at 23°C and ending at 26°C. Such transitions correspond to a smaller cooperative unit and indicate a perturbed bilayer structure. These liposomes do exhibit temperature-dependent hemolytic activity. As is shown in Fig. 7, little hemolysis is observed below the phase transition region. At an incubation temperature of 26°C, however, the vesicles become highly hemolytic. It is evident from these results that the intermembrane events that lead to hemolysis cannot, at least in this system, occur if one of the membranes is in a highly ordered state.

Observations of Liposome-Erythrocyte Adhesion by Light and Electron Microscopy

Erythrocytes aggregated in the test tube by nonlytic, L-SA liposomes form rather dense aggregates within which little detail can be seen by light microscopy. On the other hand, when erythrocytes are spread at the air-water interface and the resulting ghosts treated with agglutinating liposomes, evidence for strong adhesion is readily observed. As shown in Fig. 8 A, the membranes of such ghosts are tightly apposed and some of the larger liposomes may be seen adhering to their surfaces.

Electron microscopy reveals that the apposition of liposome and cell membrane is very close. Fig. 8 B is an electron micrograph of erythrocytes with a number of adhering liposomes. To make them identifiable, these liposomes contained, in addition to L and SA, a lipid antigen (DNP-Cap-PE) and were labeled with a ferritin-conjugated antibody *subsequent* to their interaction with the erythrocytes (see Materials and Methods for details). Liposomes of relatively low surface potential produce only slight distortion of the erythrocyte membrane as a result of attachment, although the larger vesicles sometimes flatten out on the cell membrane surface. In contrast, liposomes bearing a high surface potential (over 60 mV) often cause indentation of the cell surface at the point of attachment, and sometimes the cell membrane partially wraps around the liposome.

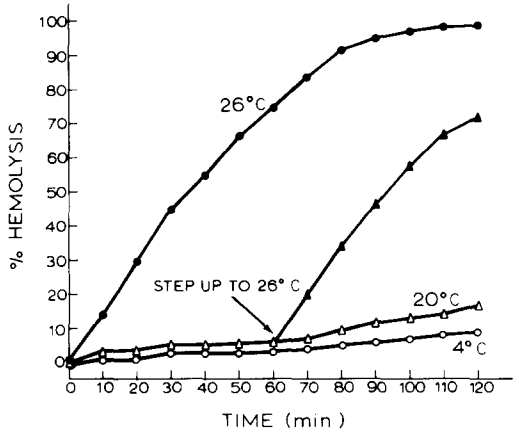

FIGURE 7 Time-course of hemolysis for DML + LL + SA liposomes above (26°C) and below (20°C) the lipid phase transition temperature. Liposomes composed of 15% LL, 2% SA, and 83% DML undergo a crystal to liquid-crystalline phase change in the temperature range 23°–26°C. Hemolysis of sheep erythrocytes in the presence of such liposomes was measured at incubation temperatures of 26°C (filled circles), 20°C (open triangles), and 4°C (open circles). The filled triangles represent an experiment in which the cells were incubated with liposomes at 20°C for 1 h, and then the temperature was raised to 26°C for an additional 60 min.

Requirement of Liposome-Cell Contact for Hemolysis

Previous experiments suggested that close contact between liposomes and erythrocytes is required for the hemolytic reaction. To test this hypothesis, liposomes containing 15% LL, 10% SA, 10% DNP-Cap-PE, and 65% L were treated with antibodies (rabbit anti-DNP serum followed by ferritin-conjugated goat anti-rabbit IgG, see Materials and Methods) *before* their interaction with cells. Such treatment severely inhibited the hemolytic activity of these liposomes. Although the antibody treatment caused a slight reduction in the liposome zeta potential from 50 to 35 mV, this does not account for the observed loss of hemolytic potential; control liposomes which had not been pretreated with ferritin antibody, but which were prepared with slightly less SA to yield zeta potentials of 35 mV, were at least 10 times more hemolytic than the treated liposomes. Electron microscopy of cells treated with the ferritin-labeled liposomes revealed that, although such vesicles attach to cells, the layer of ferritin and antibody molecules prohibits the close juxtaposition of the liposome and erythrocyte membranes.

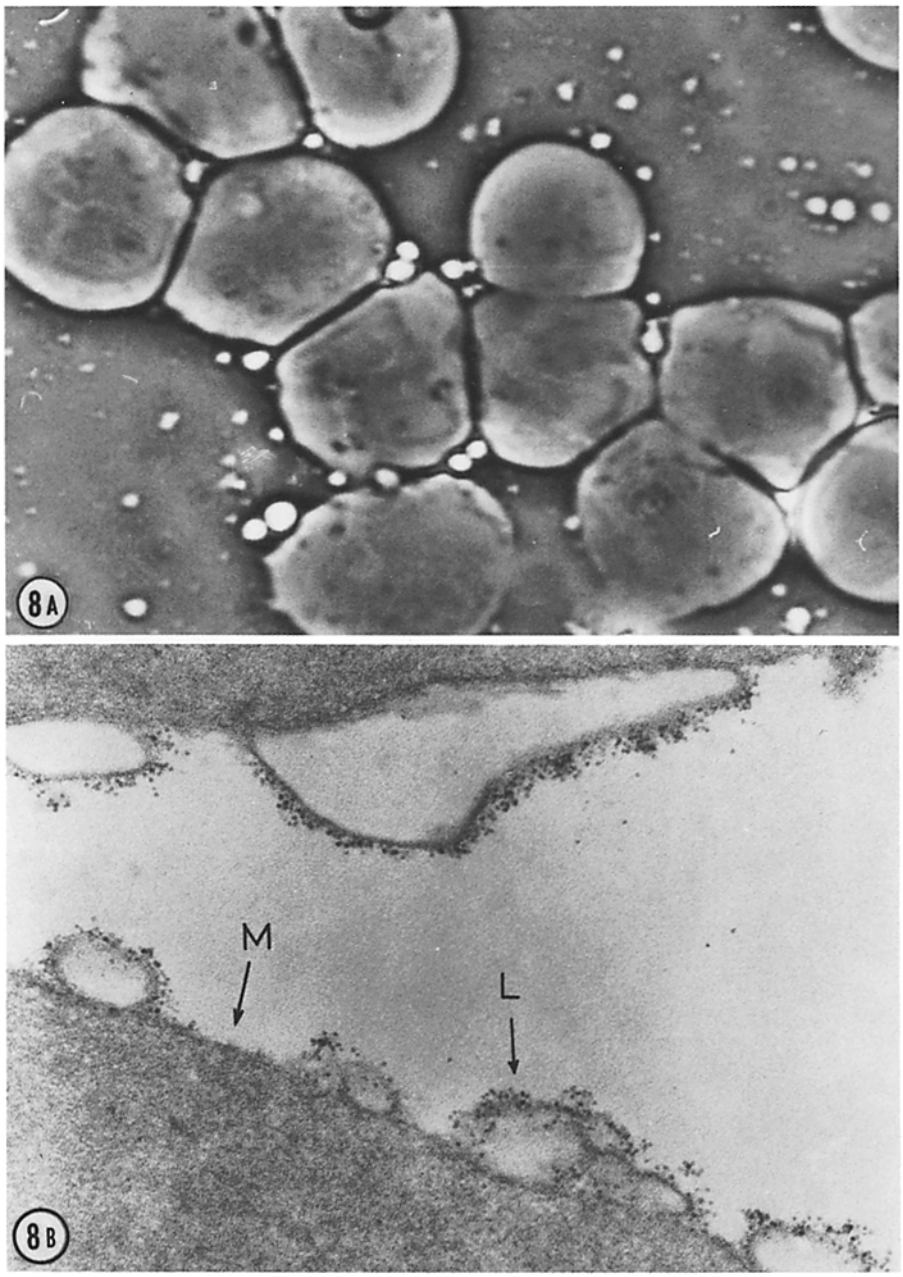

FIGURE 8 Microscope examination of liposome-erythrocyte interaction. (A) Phase micrograph of erythrocyte ghosts lysed at the air-water interface and treated with SA-containing liposomes. The liposomes appear as small bright spherules. Many of the largest of these may be seen adhering to cell surfaces. The smaller ones are obscured by the phase halo around the cells. Ghosts prepared in the same way except for the liposome treatment are spherical and unaggregated. × 1600. (B) Electron micrograph of a thin section through erythrocytes treated with liposomes composed of 10% DNP-Cap-PE, 5% SA, and 85% L for 5 min at 37°C in 0.3 M sucrose containing 0.01 M tricine buffer (pH 7.5). After liposome treatment, the cells were treated with rabbit anti-DNP serum and ferritin-conjugated goat anti-rabbit IgG, washed, fixed in glutaraldehyde and osmium, and prepared for electron microscopy as described in reference 20. The erythrocyte plasma membrane is labeled *M*, and liposomes are indicated by *L*. × 110,000.

Aside from the layer of ferritin between the vesicle and the cell membrane, the appearance of these lytic vesicles is much the same as that of the nonlytic vesicles of Fig. 8 B.

DISCUSSION

In general, the use of model systems in the study of complex physiological phenomena is predicated on the assumption that the interpretation of results is less ambiguous the fewer the variables. The most successful model systems reduce the number of variables to the minimum number necessary to mimic the desired phenomenon. Accordingly, the present study sought to modify the simplest analogue of a natural membrane, the lipid bilayer, by including in it the smallest number of molecules of defined structure that would yield a particle competent to agglutinate and lyse erythrocytes. According to the tests we have applied, the behavior of the model membrane is operationally identical to that of a typical parainfluenza-type virus particle.

The principal results of this study can be summarized as follows: (*a*) the agglutination of erythrocytes by liposomes requires that the vesicles be positively charged; neutral and negatively charged liposomes bind poorly to cell membranes. (*b*) Lysis of erythrocytes by liposomes requires that (*i*) the liposome and cell membrane be in close juxtaposition, (*ii*) both of the interacting membranes be in a fluid state, and (*iii*) the vesicle membrane contain a bilayer perturbing molecule.

Cell Agglutination: Liposome Attachment

That attachment of liposomes to cell surfaces is an electrostatic phenomenon is indicated by a number of observations: the HA titer of positively charged liposomes increases with increasing magnitude of the algebraic difference in zeta potential between the liposome and cell membrane; uncharged and negatively charged liposomes exhibit no evidence of adhering to cell surfaces; increasing electrolyte concentration diminishes the adhesion of positive liposomes to cells; positively charged erythrocytes are not agglutinated by positively charged liposomes.

In a number of experiments, we have demonstrated a linear relationship between the log of the HA titer and zeta potential difference (liposome minus erythrocyte). When examined in terms of the electrostatic forces between surfaces, this kind of relationship is predicted. The free energy change associated with the adhesion of liposomes to the surface of cells will consist of an electrostatic term (ΔG^e) and a concentration term (ΔG^c). The former will represent the energy change involved in bringing positive and negative charges together, and the latter the energy change due to the change of concentration of liposomes as they become concentrated at the cell surface. The total free energy change may then be written as:

$$\Delta G^{total} = \Delta G^c + \Delta G^e.$$

At equilibrium,

$$\Delta G^{total} = 0,$$

so,

$$0 = \Delta G^c + \Delta G^e. \tag{1}$$

Since ΔG^c is the free energy to transport 1 mol of liposomes from the bulk solution at a concentration C_b to the erythrocyte surface at a concentration of C_s, it is given by:

$$\Delta G^c = RT \ln \frac{C_s}{C_b}.$$

The total concentration ($C_s + C_b$) at the end point in a Salk assay is the concentration in the first well (C_o) divided by the hemagglutination titer, i.e., C_o/HA, and because the C_s is negligible with respect to the bulk concentration (the number of liposomes considerably exceeds the number of erythrocytes, even at the end point),

$$\Delta G^c = RT \ln \frac{C_s}{C_o/\mathrm{HA}}. \tag{2}$$

Parsegian and Gingell (28) have given an expression for the electrostatic term which, under the conditions appropriate to our experiments, namely that the distance of separation of the two membranes is small compared to the Debye length, reduces to:

$$\Delta G^e = k\psi_1\psi_2, \tag{3}$$

where k is a proportionality constant and the ψ's represent the potentials of the membrane surfaces. If $\psi_1 = \psi_{rbc}$ is the surface potential of the erythrocytes and $\psi_2 = \psi_{lip}$ is the potential of the liposomes, then combination of equations 1-3 yields,

$$k\psi_{rbc}\psi_{lip} = -RT \ln \frac{C_s \mathrm{HA}}{C_o} . \quad (4)$$

This can be rewritten in derivative form as:

$$\frac{d\psi_{lip}}{d(\ln \mathrm{HA})} = -\frac{RT}{k\psi_{rbc}} - \frac{RT}{k\psi_{rbc}} \frac{d(\ln(C_s/C_o))}{d(\ln \mathrm{HA})} . \quad (5)$$

Since C_o and ψ_{rbc} are constant for experiments represented by the closed circles of Fig. 1, and since a HA end point is expected to represent a point where the ratio of the number of agglutinating species to erythrocytes is a particular value (probably near unity), the second term of equation 5 vanishes and this simple treatment predicts the observed linear relationship between liposome zeta (≅surface) potential and HA titer. If ψ_{rbc} is varied and ψ_{lip} held constant, equation 5 (after exchanging ψ_{rbc} and ψ_{lip}) describes the data represented by open circles of Fig. 1 as well.

Liposome-Induced Cytolysis

Our observation that neutral vesicles, even containing large proportions of LL, are hemolytically inactive suggests that simple collision of liposomes with cells is insufficient to cause lysis. Furthermore, apposition of liposome and cell membranes must be close; intervention of a ferritin-conjugated antibody is sufficient to greatly inhibit hemolysis. Given appropriate characteristics of the liposome membrane, adsorption is followed by lysis.

Experiments on the capacity of SA + LL + DML liposomes to induce hemolysis indicate that membrane fluidity is a crucial factor in the lysis reaction. Liposomes incubated with erythrocytes below T_c adsorb quite effectively to the cells, but do not cause hemolysis. As the temperature is raised through T_c, however, the cells begin to lyse.

In addition to a fluid membrane, hemolytic activity requires that liposomes contain a proportion of a bilayer perturbant species. In these experiments, we use LL which, as Haydon and Taylor (9) pointed out, is wedge-shaped and, consequently, poorly accommodated by a lipid bilayer. As a consequence of this shape, LL is a micelle-forming surfactant and bilayer membranes treated with high concentrations themselves become micellar and disaggregate (3). This extreme effect would, of course, cause cell lysis, but LL is lytic at concentrations much lower than those necessary to dissolve membranes, and it is probable that in most situations lysis is a result of increased permeability to small molecules and ions (17). In any case, the basis of lysis by liposomes must be osmotic, since, as shown in the following paper, when erythrocytes are treated with liposomes in the presence of dextrans, the cells undergo fusion rather than lysis (20). Were it merely a question of the erythrocyte membrane dissolving under the influence of LL, the presence of an osmotic stabilizer would have no such effect.

Given that the integrity of the erythrocyte membrane is largely preserved during interaction of liposomes with cell membranes, there are still at least three possible mechanisms that could account for our results: (*a*) LL could diffuse from an attached liposome into the adjacent cell membrane. The resulting increase in ion and small molecule permeability would then produce colloid osmotic swelling. (*b*) Fusion of liposomes with cell membranes could introduce a patch of liposome membrane that is leakier than the original cell membrane. Since liposomes containing up to the highest concentration of LL we have used remain virtually impermeable to hemoglobin, such a patch would be leaky primarily to ions and small molecules, and the cell would burst from entry of water. (*c*) The liposome may fuse with the cell membrane, and the fusion process could itself generate a large enough permeability change to produce osmotic lysis. In this case, the requirement for LL in active liposomes would relate to its being a prerequisite for fusion.

Evidence is presented in a following paper that liposomes active in hemolysis do fuse with cell membranes (21). In addition, it is known that liposomes containing LL are rather leaky to small molecules and ions (13, 17). Given these prerequisites, then, it may be assumed that at least mechanism (*b*) is operative. Mechanism (*a*) appears to be inconsistent with the inhibition of hemolysis by DNP liposomes caused by anti-DNP. If LL exchanged readily between liposomes and cell membranes, it seems rather unlikely that the intervention of a water layer only a few nm thick would prevent it. Mechanism (*a*) cannot be entirely rejected, however, since there is evidence for transfer of phospholipids from liposomes to cell membranes (11, 24) and to other liposomes (19). Mechanism (*c*) would be very difficult to differentiate from mechanism (*b*) since the latter would also be transitory, the incorporated patch of liposome becoming elimi-

nated through interdiffusion of the membrane and liposome lipids.

Electron microscopy of cells that have been lysed with DNP-containing liposomes and then treated with ferritin-conjugated anti-DNP reveals that ferritin is often associated with patches of the cell membrane that are broken. Such observations suggest that, regardless of the mechanism leading to lysis, those areas of the cell membrane that have acquired lipid from liposomes are weak points which, as the cell swells, rend first.

This investigation was supported by U. S. Public Health Service grant AI 11428.

Received for publication 11 December 1975, and in revised form 3 May 1976.

REFERENCES

1. BANGHAM, A. D., R. FLEMANS, D. H. HEARD, and G. V. SEAMAN. 1958. An apparatus for the microelectrophoresis of small particles. *Nature (Lond.).* **182:**642–644.
2. BANGHAM, A. D., M. W. HILL, and N. G. A. MILLER. 1974. Preparation and use of liposomes as models of biological membranes. *In* Methods in Membrane Biology, Vol. I. E. D. Korn, editor. Plenum Publishing Corporation, New York. Chap. 1.
3. BANGHAM, A. D., and R. W. HORN. 1964. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol.* **8:**660–668.
4. BARTON, P. G. 1968. The influence of surface charge density of phosphatides on the binding of some cations. *J. Biol. Chem.* **243:**3884–3890.
5. BATZRI, S., and E. D. KORN. 1975. Interaction of phospholipid vesicles. Endocytosis and fusion as alternate mechanisms for the uptake of lipid-soluble and water-soluble molecules. *J. Cell Biol.* **66:**621–634.
6. DAVIES, J. T., and E. K. RIDEAL. 1963. Interfacial Phenomena. Academic Press, Inc., New York. Chap. 3.
7. GRANT, C. W., and H. M. MCCONNELL. 1973. Fusion of phospholipid vesicles with viable *Acholeplasma laidlawii*. *Proc. Natl. Acad. Sci. U. S. A.* **70:**1238–1240.
8. GREGORIADIS, G., and E. D. NEERUNJUN. 1975. Homing of liposomes to target cells. *Biochem. Biophys. Res. Comm.* **65:**537–544.
9. HAYDON, D. A., and J. TAYLOR. 1963. The stability and properties of bimolecular lipid leaflets in aqueous solutions. *J. Theor. Biol.* **4:**281–296.
10. HOWE, C., and C. MORGAN. 1969. Interactions between Sendai virus and human erythrocytes. *J. Virol.* **3:**70–81.
11. HUANG, L., and R. E. PAGANO. 1975. Interaction of phospholipid vesicles with cultured mammalian cells. I. Characteristics of uptake. *J. Cell Biol.* **67:**38–48.
12. INBAR, M., and M. SHINITZKY. 1974. Increase of cholesterol level in the surface membranes of lymphoma cells and its inhibitory effect on ascites tumor development. *Proc. Natl. Acad. Sci. U. S. A.* **71:**2128–2130.
13. INOUÉ, K., and T. KITAGAWA. 1974. Effect of exogenous lysolecithin on liposomal membranes and its relation to membrane fluidity. *Biochim. Biophys. Acta.* **363:**361–372.
14. JOHNSON, M. E., S. SIMON, J. W. KAUFFMAN, and R. C. MACDONALD. 1973. A synthetic lecithin containing branched-chain fatty acids: physical properties and membrane studies. *Biochim. Biophys. Acta.* **291:**587–594.
15. KORN, E. D., B. BOWERS, S. BATZRI, S. R. SIMONS, and B. J. VICTORIA. 1974. Phospholipids and membrane fusion in endocytosis and exocytosis. *J. Supramol. Struct.* **2:**517–528.
16. MAGEE, W. E., C. G. GOFF, J. SCHOKNECHT, M. D. SMITH, and K. CHERIAN. 1974. The interaction of cationic liposomes containing entrapped horseradish peroxidase with cells in culture. *J. Cell Biol.* **63:**492–504.
17. MANDERSLOOT, J. G., F. C. REMAN, L. M. VAN DEENEN, and J. DE GIER. 1975. Barrier properties of lecithin/lysolecithin mixtures. *Biochim. Biophys. Acta.* **382:**22–26.
18. MARTIN, F., and R. MACDONALD. 1974. Liposomes can mimic virus membranes. *Nature (Lond.).* **252:**161–163.
19. MARTIN, F. J., and R. C. MACDONALD. 1976. Phospholipid exchange between phospholipid bilayer vesicles. *Biochemistry.* **15:**321–327.
20. MARTIN, F. J., and R. C. MACDONALD. 1976. Lipid vesicle-cell interactions. II. Induction of cell fusion. *J. Cell Biol.* **70:**506–514.
21. MARTIN, F. J., and R. C. MACDONALD. 1976. Lipid vesicle-cell interactions. III. Introduction of a new antigenic determinant into erythrocyte membranes. *J. Cell Biol.* **70:**515–526.
22. NEURATH, A. R., S. K. VERNON, R. W. HARTZELL, and B. A. RUBIN. 1972. Haemolysis by Sendai virus: lack of requirement for neuraminidase. *J. Gen. Virol.* **16:**245–249.
23. PAGANO, R. E., and L. HUANG. 1975. Interaction of phospholipid vesicles with cultured mammalian cells. II. Studies of mechanism. *J. Cell Biol.* **67:**49–60.
24. PAGANO, R. E., L. HUANG, and C. WEY. 1974. Interaction of phospholipid vesicles with cultured mammalian cells. *Nature (Lond.).* **252:**166–167.

25. PAPAHADJOPOULOS, D., E. MAYHEW, G. POSTE, S. SMITH, and W. VAIL. 1974. Incorporation of lipid vesicles by mammalian cells provides a potential method of modifying cell behavior. *Nature (Lond.)*. **252:**163–166.
26. PAPAHADJOPOULOS, D., G. POSTE, and E. MAYHEW. 1974. Cellular uptake of cyclic AMP captured within phospholipid vesicles and effect on cell-growth behavior. *Biochim. Biophys. Acta.* **363:**404–418.
27. PAPAHADJOPOULOS, D., G. POSTE, and B. E. SCHAEFFER. 1973. Fusion of mammalian cells by unilamellar lipid vesicles: influence of lipid surface charge, fluidity and cholesterol. *Biochim. Biophys. Acta.* **323:**23–42.
28. PARSEGIAN, V. A., and D. GINGELL. 1972. On the electrostatic interactions across a salt solution between two bodies bearing unequal charges. *Biophys. J.* **12:**1192–1204.
29. SALK, J. E. 1944. A simplified procedure for titrating hemagglutinating capacity of influenza-virus and the corresponding antibody. *J. Immunol.* **49:**87–98.
30. TENFORDE, T. 1970. Microelectrophoretic studies on the surface chemistry of erythrocytes. *Adv. Biol. Med. Phys.* **13:**43–105.
31. WEISSMANN, G., D. BLOOMGARDEN, R. KAPLAN, C. COHEN, S. HOFFSTEIN, J. COLLINS, A. GOTTLIEB, and D. NAGLE. 1975. A general method for the introduction of enzymes by means of immunoglobulin-coated liposomes into lysosomes of deficient cells. *Proc. Natl. Acad. Sci. U. S. A.* **72:**88–92.
32. WIRTZ, K. W. A. 1974. Transfer of phospholipids between membranes. *Biochim. Biophys. Acta.* **344:**95–117.
33. YI, P. N., and R. C. MACDONALD. 1973. Temperature dependence of the optical properties of aqueous dispersions of phosphatidylcholine. *Chem. Phys. Lipids.* **11:**114–134.