Phospholipid Exchange between Bilayer Membrane Vesicles†

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ABSTRACT: The turbidity of lipid vesicles, freshly prepared by sonicating purified dimyristoyllecithin (DML) in dilute KCl solutions, was measured as a function of time at various temperatures. A sharp maximum in the rate of increase of turbidity is found just above the crystal:liquid-crystal phase transition temperature (T_m). The initial rate of turbidity increase is first order with respect to DML concentration. Electron and light microscopy reveal large vesicles which are not present before incubation or after incubation at temperatures far from the T_m. When temperature, rather than time, is the independent variable, a sharp drop in turbidity is seen at the T_m. The magnitude of this drop and the temperature at which it occurs were used to measure the rate of lipid transfer between vesicles composed of different lipids. A mixture of DML vesicles and dipalmitoyllecithin (DPL) vesicles exhibits sharp drops in turbidity at 24 and 41°, the corresponding T_m's. With time, the magnitude of the transition at 24° decreases while that which was originally at 41° moves to lower temperatures and increases in magnitude. At equilibrium there is a single transition at 32.5° characteristic of vesicles composed of equimolar DPL and DML. The rate at which equilibrium is approached increases at around 24° and again around 41°. These observations indicate that vesicles are in equilibrium with monomolecular lipid, the concentration of the latter being higher the shorter the lipid acyl group or the smaller the vesicle. DML molecules are therefore lost from small vesicles to large vesicles (DML system) or lost from DML vesicles to DML-DPL vesicles (mixed system). When DML vesicles containing a few percent brain gangliosides were studied, different behavior was observed; the initial rate of increase of turbidity becomes second order in lipid concentration, and the rate constant increases with increasing concentrations of KCl. The kinetic order, coupled with the fact that electrolyte reduces intervesicle electrostatic repulsion, argues that in this situation the mechanism of vesicle growth requires vesicle collision.

Lipid exchange between membranes has been found to occur in a number of situations. The exchange of phospholipid molecules between natural membranes in vitro has been demonstrated by Wirtz (1974), McMurray and Dawson (1969), Wojtczak et al. (1971), and Akiyama and Sakagami (1969), and between artificially prepared phospholipid aggregates and the membranes of subcellular organelles by Zilversmit (1971). A protein isolated from beef heart and liver is known to accelerate these processes (Wirtz and Zilversmit, 1969). Recent evidence indicates that lipid molecules also exchange between bilayer vesicles and the surface membranes of cells. In fact, liposomes are quickly gaining favor as a means of introducing foreign molecules into intact cell membranes. Cholesterol (Inbar and Shinitzky, 1974) and phospholipid (Grant and McConnell, 1973; Pagano et al., 1974; Papahadjopoulos et al., 1974a), for example, have been inserted into the plasma membranes of viable cells by means of liposome treatment. At present, however, the mechanism(s) of transfer in these systems is not well understood. There is evidence for both liposome-cell membrane fusion (Grant and McConnell, 1973; Pagano et al., 1974; Papahadjopoulos et al., 1974a) and for simple molecular exchange (Demel et al., 1973; Pagano and Huang, 1975).

The study of interaction between artificial model membranes represents a simplified approach to the basic problem of membrane-membrane interactions and has shed some light on the question of intermembrane molecular exchange. It is known, for example, that small (300 Å), single compartment vesicles form larger structures in periods of several hours when incubated at appropriate temperatures (Taupin and McConnell, 1972). Taupin and McConnell (1972) were the first to suggest that this process involves liposome-liposome fusion. Papahadjopoulos et al. (1974b), Prestegard and Fellmeth (1974), and Kantor and Prestegard (1975) have provided additional evidence in favor of fusion. Membrane fusion has also been proposed to account for the transfer of proteins (Drachev et al., 1974) and fluorescent probes (Pohl et al., 1973) from liposomes to planar bilayers. However, much of the evidence in favor of a fusion mechanism is indirect and can just as reasonably be interpreted in terms of simple molecular exchange of the type described by Pagano and Huang (1975).

The present study was undertaken in order to elucidate the molecular mechanism of intermembrane phospholipid exchange in several simple and well-defined situations. In the first series of experiments, the process whereby small, unilamellar dimyristoyllecithin (DML)[1] vesicles become larger was examined with respect to temperature dependence and apparent rate constant. In the second series of experiments, the transfer of phospholipid molecules between DML and dipalmitoyllecithin (DPL) vesicles was examined as a function of temperature and incubation time. The unidirectional transfer observed in the second set of experiments and the kinetic order (first) found in the first set indicate that DML vesicles are in equilibrium with a low concentration of monomeric DML and that the latter are progressively inserted into large DML or mixed DML-DPL

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[1] Abbreviations used are: DML, dimyristoyllecithin; DPL, dipalmitoyllecithin; T_m, phase transition midpoint temperature.

vesicles. In both situations, a dramatic increase in rate was observed at the relevant lipid phase transition temperature. Evidence indicative of fusion of lipid bilayers was found only in one situation where the membranes contained lipids (gangliosides), in addition to lecithin.

Materials and Methods

Dimyristoyllecithin (1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine) and dipalmitoyllecithin (1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine) were synthesized in this laboratory using methods previously described, except that the fatty acid salt was formed with triethylamine (Johnson et al., 1973), and purified by silica gel chromatography using standard methods. These lipids were, by thin-layer chromatography, free of detectable impurities. In contrast to commercial products described elsewhere (Yi and MacDonald, 1973) these lipids did not exhibit optically detectable pretransition events. Microelectrophoresis of dispersions of DML and DPL revealed less than 0.3% fatty acid contamination, based on calculations using electrical double layer theory. Purity was not assayed following experiments, but the sharpness of the phase transitions of controls indicates that decomposition was negligible. Gangliosides were extracted from beef brain and purified as described by Radin (1969) except that barium precipitation was omitted. For preparation of liposomes, weighed portions of lipid were dispersed in twice-distilled water which contained 10^{-4} *M* EDTA and, in some cases, KCl. When lipid mixtures were used, the lipids were combined in chloroform–methanol (4:1) which was subsequently removed under reduced pressure. The resulting lipid film was dispersed in the appropriate aqueous medium. Dispersions were irradiated for a few minutes with a probe type ultrasonic apparatus.[2] Samples were not cooled during sonication to assure that the temperature rose above that of the phase transition of the relevant lipid. Optical densities were typically 0.25 for DML sonicated in 0.1 *M* KCl at 10 mg of lipid/ml.

The initial rate of DML vesicle growth was taken as proportional to the initial rate of change of optical density. The latter was measured as follows. Immediately after sonication, the DML dispersion was quickly cycled through the phase transition by alternately plunging the sample into warm and ice water. After two such cycles, the sample was taken from the ice bath and placed in a water-jacketed cell in a spectrophotometer. Water at the temperature appropriate to the experiment was circulated through the cell jacket sufficiently before the addition of the sample that the cell and cell holder had equilibrated thermally. When the sample temperature reached that of the jacket (approximately 30 sec), a recorder connected to the spectrophotometer was switched on and a record of optical density as a function of time was obtained. Although the slopes of such traces were nearly linear for several minutes, the initial rate was routinely taken as the slope of the line (in OD units/min) drawn through points at 30 and 60 sec. Samples of multiplicate experiments were sonicated to the same initial optical density. The wavelength for all optical measurements was 400 nm.

Phase transitions were determined by measuring absorbance of lipid dispersions as a function of temperature (Yi and MacDonald, 1973). Electron microscopy of negatively stained preparations was done essentially as described by Bangham and Horne (1964).

[2] It should be understood that the effect produced by sonication for a given duration of time depends markedly on the instrument and conditions.

Results

Liposome model membranes undergo a first-order phase transition from a crystalline to a liquid-crystalline state at a characteristic temperature (T_m). For a membrane composed of a single lipid, this melting occurs within a narrow range and at a temperature characteristic of the acyl moieties (Ladbrooke and Chapman, 1969). As shown previously, aqueous dispersions of phospholipid exhibit a decrease in turbidity at the T_m (Abramson, 1971; Yi and MacDonald, 1973). In addition to being a convenient means of determining the value of T_m, the magnitude and sharpness of this turbidity drop, when plotted vs. temperature, can be used to measure the purity, and, in the case of mixed dispersions, the relative amount of a lipid. The turbidity of liposome dispersions is also a measure of the average size of the particles, larger liposomes or liposome aggregates scattering more light than smaller ones (Bangham et al., 1967). In the following experiments changes in the optical properties of aqueous lipid dispersions were used to follow (a) the rate of increase in DML vesicle size and (b) the exchange of lipid between DML and DPL vesicles.

DML Liposomes Become Smaller upon Sonication but Larger upon Incubation. When DML is sonicated in aqueous solution, the optical density of the dispersion decreases, corresponding to a reduction in size of the light scattering unit. When a sonicated dispersion is incubated at constant temperature, its optical density increases at a rate dependent upon lipid concentration and temperature. As will be seen below, the increase of optical density is optimal at temperatures slightly above the crystal:liquid-crystal phase transition temperature. The transition temperature of DML is 24° (Chapman et al., 1967).

The opposite effects of sonication and incubation on optical density suggest that small vesicles formed during sonication are replaced by larger structures during incubation at a temperature slightly above T_m. If this is the case, the effect of incubation should be reversible by resonication. This is, in fact, observed. Indeed, dispersions may be cycled indefinitely between high and low turbidities by alternating sonication with incubation.

Since light scattering does not distinguish between aggregation of small vesicles and the formation of large vesicles from smaller ones, the dispersions were examined under the electron microscope before and after incubation. The diameters of vesicles of a freshly sonicated dispersion of DML range from 20 to 60 nm. The same distribution of vesicle sizes was observed in a dispersion that had been allowed to stand at 4° for 1 hr. In contrast, when sonicated vesicles were incubated at 26° for 1 hr, their diameters became significantly larger, ranging from 40 to several hundred nm. In addition, dispersions subjected to these conditions have been examined under the light microscope; with phase contrast optics nothing is visible in a freshly sonicated dispersion, but after incubation at 26° many vesicles large enough to be clearly resolved are seen. The latter are not aggregated. Whether the larger vesicles formed upon incubation are uni- or multilamellar is not known.

Temperature Dependence of DML Vesicle Growth. The rate of increase in DML vesicle size varies dramatically with incubation temperature. In Figure 1 the initial rate of optical density increase for a DML dispersion is plotted against incubation temperature. Outside the temperature

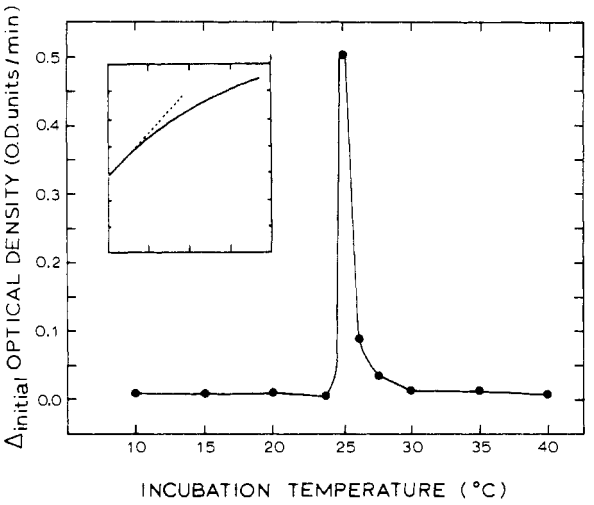


FIGURE 1: Initial rate of DML vesicle growth as a function of temperature. The initial rate of optical density change of a sonicated dispersion of lipid is plotted against incubation temperature. Initial rates represent the slope, at short times, of OD recorded as a function of time upon quickly raising the sample temperature from near 0° to that shown on the abscissa. Such a curve is shown in the inset, the dotted line representing the initial slope. The ordinate and abscissa of the inset are divided into units of 0.1 OD unit and 5 min, respectively.

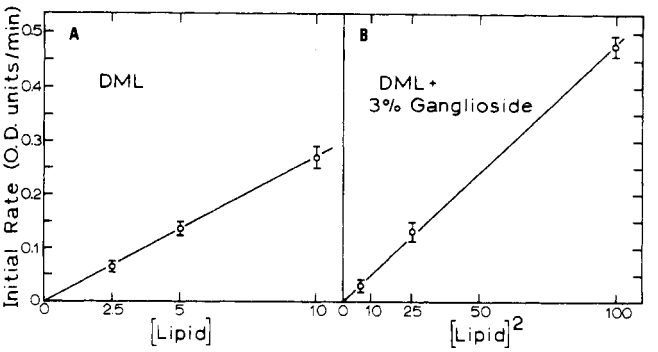


FIGURE 2: Kinetics of vesicle growth. The initial rate of optical density change (proportional to the rate at which the vesicles increase in size), expressed as OD units/min, is plotted against lipid concentration (in mg/ml) for dispersions composed of purified DML (a) and DML containing 3% (w/w) gangliosides (b). For the pure DML vesicles, the abscissa represents lipid concentration whereas for the ganglioside-containing vesicles it represents the square of the lipid concentration. Bars represent standard deviations for three experiments.

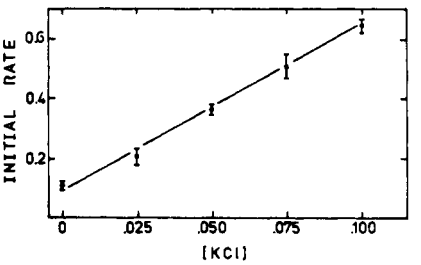


FIGURE 3: Rate of DML vesicle growth as a function of KCl concentration. Vesicles contained 3% gangliosides. The ζ potential for such vesicles in distilled water was about −50 mV and in 0.1 *M* KCl about −5 mV. The initial rate of change in optical density was measured as described in the legend to Figure 1. The bars represent standard deviations of three experiments.

range 24–30°, the rate is very slow; within this range there is a dramatic increase in rate. The maximum rate, occurring at 25°, represents a 100–200-fold increase over the rate at temperatures below 24° or above 30°.

Reaction Kinetics. The kinetic order of the process of DML vesicle growth was determined from the relationship between lipid concentration and the initial rate of optical density change at 26°. Figure 2a shows that the rate of increase in optical density (OD units/min) for a dispersion of purified DML suspended in 0.1 *M* KCl follows first-order kinetics, i.e., the rate is linearly related to the lipid concentration.

DML-ganglioside vesicles under identical conditions, on the other hand, exhibit a different reaction order; Figure 2b shows that reaction rates for dispersions of DML containing 3% ganglioside (w/w) correspond to second-order kinetics, that is, the initial rates are proportional to the square of the lipid concentration. Since gangliosides are acidic (carboxyl), these vesicles are negatively charged.

If Raleigh scattering is obeyed, then the rate of vesicle growth will be proportional to the rate of change of optical density. Since this condition is approximately met by highly sonicated lipid dispersions (the particle scattering factor does not decrease more than 10% until vesicle diameters exceed 50 nm; Yi and MacDonald, 1973), turbidity changes provide a reasonably accurate measure of vesicle growth rate. For this reason, as well as to simplify the kinetic analysis (see Discussion), we have restricted our measurements to initial rates. The possibility is recognized that a few quite large liposomes may remain in a dispersion even after thorough sonication. Such vesicles will make a disproportionate contribution to the total optical density but a negligible contribution to optical density *changes*.

Effect of Monovalent Salt on the Rate of Growth of Ganglioside-containing Vesicles. Since the kinetics in the case of DML vesicles containing 3% ganglioside suggest the possibility of a process involving vesicle collision, it was of interest to determine whether an increase in the surface potential of the vesicles, and hence the electrostatic barrier to membrane approach, would diminish the rate of increase in optical density. As seen in Figure 3, when the electrolyte (KCl) concentration is reduced from 0.1 *M* to 0, the initial rate of change of optical density drops linearly to 0.1 OD unit/min. The ζ potential of these vesicles increases from less than −5 to over −50 mV with this change in electrolyte concentration. This potential change corresponds to a several-fold change in electrical repulsion energy at vesicle–vesicle contact (Parsegian and Gingell, 1972). Increased electrical repulsion is therefore easily able to account for the data presented in Figure 3.

Lipid Transfer between DML and DPL Vesicles. In the next series of experiments the transfer of phospholipid molecules between two populations of vesicles, one composed of DML and the other of DPL, is examined.

Since the method relies upon turbidimetric measurement of lipid phase transition parameters, its basis will be briefly described. If the optical density of a dispersion of a synthetic lipid is measured as a function of increasing temperature, a sharp drop is seen at the phase transition temperature (Abramson, 1971). This drop results from a change of the isotropic and anisotropic components of the refractive index of the lipid bilayer wall of the vesicles (Yi and MacDonald, 1973). As long as the sample is sufficiently dilute or well sonicated that multiple scattering is negligible, the ratio of the optical density just above to the optical density just below the transition temperature (these temperatures are chosen to minimize the influence of the linear decrease of OD outside the transition region) is a constant that depends

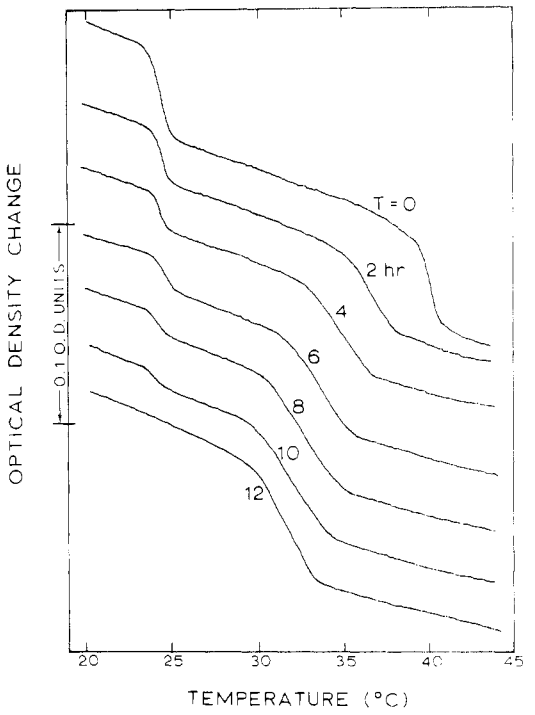


FIGURE 4: Change in optical density as a function of temperature for a mixed dispersion of DML and DPL vesicles following various times of incubation at 50°. DML and DPL were dispersed separately at concentrations of 10 mg/ml in distilled water by sonication (final OD in both cases was 0.7). Equal volumes of the two solutions were added to a cuvette and the mixed dispersion was incubated at 50°. Temperature vs. turbidity scans were taken at 2-hr intervals. Scanning rate was 2°/min.

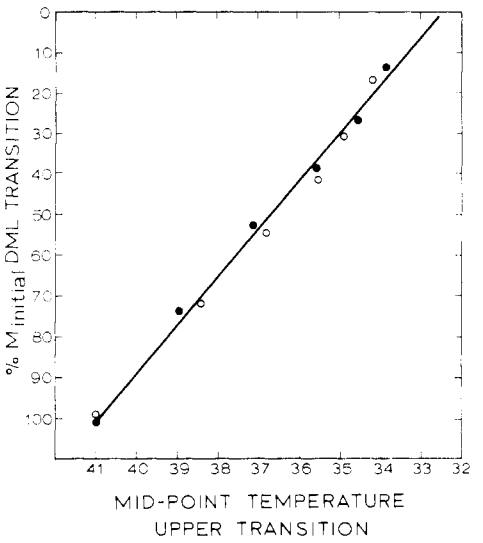


FIGURE 5: Percent of the initial magnitude of the DML transition plotted vs. the midpoint temperature of the upper transition. A mixed dispersion of DML and DPL vesicles was prepared as described in the legend to Figure 4. The mixture was incubated at 50° for 15 hr and the magnitude of the DML transition as well as the temperature of the upper transition (Figure 4) was determined at 2-hr intervals. Open and filled circles represent data from duplicate experiments.

upon the lipid. For DML, the transition temperature of which is 24°, $OD^{25°}/OD^{23°}$ = 0.77 (this work) and for DPL, the transition temperature of which is 41°, $OD^{42°}/OD^{40°}$ = 0.75 (Yi and MacDonald, 1973). The magnitude of the optical density drop at the transition temperature depends upon the number of lipid molecules participating in the phase transition and so, for a population of vesicles of fixed composition, is a measure of concentration.

Because vesicles composed of mixtures of DML and DPL also undergo transitions at characteristic temperatures between 24 and 41°, a record of optical density over this temperature range allows calculation of the composition of vesicles in mixtures of the two lipids. A scan of optical density for a mixture of equal amounts of DPL and DML vesicles immediately after mixing is shown in Figure 4 (trace marked T = 0). The transition of each population of vesicles is evident at 24 and at 41°. If this mixture is held at 4°, the vesicles maintain separate identities for days, but when it is incubated at 50°, there are, over a period of 12 hr, progressive changes in the OD–temperature scans. As shown in the traces of Figure 4, taken at 2-hr intervals, the DML transition remains at 24° but its magnitude diminishes to 0 while the upper transition falls in temperature from 41 to 32° with increasing magnitude and decreasing sharpness. With increasing duration of incubation then, DPL vesicles appear to acquire progressively greater numbers of DML molecules as indicated by the appearance of the new transition corresponding to vesicles composed of a mixture of DML and DPL. The DML vesicles, although retaining their identities, decrease in size, number, or both, as shown by the diminution of the 24° transition. After 4 hr of incubation, for example, two distinct populations of vesicles are present: one composed of DML, the other a mixture of DML and DPL. At this time, 30% of the original DML is present in DML vesicles while the remaining 70% has entered DPL vesicles as judged by the 6° shift from 41 to 35° of the higher transition. During the incubation period there is a linear relationship between the change in magnitude of the lower transition and the change in temperature of the upper transition. (To avoid the possibility that DML vesicle growth might obscure the measurement of DML vesicle loss, lipids for these experiments were sonicated only long enough to eliminate multiple scattering, i.e., until $OD^{25°}/OD^{23°}$ = 0.77.) This is illustrated graphically in Figure 5 in which the left axis represents the DML transition (as $\%M_{initial}$) remaining as the temperature of the midpoint of the higher transition shifts down from 41°. The open and filled circles in Figure 5 represent duplicate experiments.

Temperature Dependence of Lipid Transfer. The rate of transfer of DML molecules to DPL vesicles is strongly temperature dependent. As shown in Figure 6, for a 14-hr incubation, the rate of transfer is insignificant at temperatures below 15°. In the range 15–25° there is a sharp increase in rate. A second, even more dramatic increase in rate is evident in the range 35–45°. Thereafter, an additional increase in temperature has little effect. The transfer rate based on the loss of DML vesicles is about twice as large as that based on the shift of the upper transition for incubations in the temperature range 15–25°. It therefore appears that about half of the DML transferred in this temperature range is going into a small fraction of the DPL vesicles and that most of the latter do not acquire appreciable amounts of DML until above their transition temperature.

Discussion

The present results indicate that the increase in turbidity of a freshly sonicated dispersion of purified DML observed at room temperature is due to growth in size of the vesicles. The process follows first-order kinetics and occurs at a significant rate only at temperatures in the region of the crystal:liquid-crystal phase transition.

Apparent first-order kinetics would be found if DML vesicles were at equilibrium with a constant concentration of DML monomers ([M]). This is understood by considering the following equations:

$$[V_S] = n_S[M], \qquad K_S = [M]^{n_S}/a_S \tag{1}$$

$$[V_L] = n_L[M], \qquad K_L = [M]^{n_L}/a_L \tag{2}$$

$$V_S + (n_L - n_S)M \underset{k_b}{\overset{k_f}{\rightleftharpoons}} V_L \tag{3}$$

Equations 1 and 2 represent equilibration of vesicles with monomeric DML. Equation 3 is their sum and describes the interconversion of large and small vesicles. V represents vesicles, large (L) or small (S), and containing n_S or n_L molecules, brackets indicate concentrations, and a represents activity. The concentration of monomers is assumed small enough that the activity coefficient is one. For simplicity, large and small vesicles are assumed to be homogeneous populations. Since vesicles may be taken as a separate phase of pure material, their activities are constant. The concentrations of monomer defined by eq 1 and 2 are thus constants and represent critical micellar concentrations. The rate of growth of vesicles (r) is from eq 3:

$$r = k_f[V_S][M]^{n_L - n_S} - k_b[V_L] \tag{4}$$

Given initial rates, the second term is essentially 0 and eq 4 reduces to a rate that is first order in small vesicle concentration. The value of [M] in eq 4 will be either the critical micellar concentration of large or small vesicles, depending upon which is the largest. In either case, as long as at least some large and small vesicles are found in a dispersion, [M] will be independent of vesicle concentration. Since the amount of lipid present as monomers will be very much smaller than that in vesicles for any dispersions that exhibit appreciable turbidity, $[V_S]$ will be essentially equal to total lipid concentration, and eq 4 predicts the experimentally observed behavior of purely DML vesicles.

It should be recognized that eq 4 merely states that a dispersion containing almost entirely small vesicles will move in the direction of the equilibrium distribution at an initial rate depending upon total lipid concentration. It does not make any prediction about where that equilibrium lies. Large and small vesicles could have the same stability and correspondingly be in equilibrium with identical concentrations of monomers. In that case, the average size of vesicles would still increase and would do so with first-order kinetics; the driving force would be purely entropic. There is evidence, however, that small vesicles are relatively unstable because of the small radius of curvature requiring a different packing density on the inner and outer monolayers (Chan et al., 1973; Thompson et al., 1974). It is therefore probable that an enthalpy difference provides an important contribution to the driving force for vesicle growth.

Parenthetically, the growth of larger vesicles at the expense of smaller ones rather closely resembles Ostwald ripening, i.e., the formation of large crystals from small upon prolonged standing in a mother liquor of recrystallization.

The simple thermodynamic and kinetic arguments presented above can account for the growth of vesicles and the observed kinetic order, but not for the rate maximum near 25°. This temperature maximum must be due to the temperature dependence of the rate constants that describe monomer transfer. In general, a maximum would appear if a single rate constant were limiting and it itself exhibited a temperature maximum, or if the critical prrameter depend-

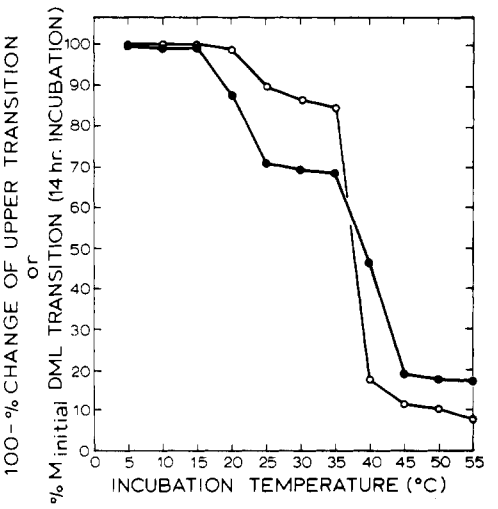
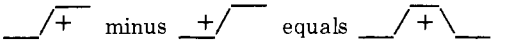


FIGURE 6: Rate of DML and DPL mixing as a function of temperature of incubation. DML and DPL vesicles were mixed as described in the legend for Figure 4 and aliquots thereof were allowed to incubate for 14 hr at various temperatures. At the end of the incubation period, the optical density as a function of temperature for each sample was obtained by scanning in the spectrophotometer between about 20 and 45°. From these scans the change in magnitude of the lower transition and the change in temperature of the midpoint of the upper transition were determined. These data are presented in the figure as a function of the incubation temperature. The left axis represents percent of the initial magnitude of the DML transition as well as a corresponding measure of the percent change of the upper transition temperature (41° taken as 0%, 32° as 100%).

ed upon the difference between two rate constants, each of which increased stepwise but at a slightly different temperature, for example:



(temperature increasing from left to right, the + marking a particular temperature). A number of structural parameters that characterize bilayers of lipids such as DML or DPL change stepwise at the phase transition temperature. These include a decrease in order (Chapman et al., 1967) and an increase in fluidity (Hubbell and McConnell, 1971), a decrease in thickness, an increase in area (Chapman et al., 1967), and a decrease in density (Träuble and Haynes, 1971). Such changes might well be expected to provoke similar changes in the kinetic coefficients that describe the exchange of lipid molecules between vesicles. Since small and large vesicles melt at somewhat different temperatures (Chan et al., 1973), the requirement for step changes in relevant kinetic parameters occurring at different temperatures would be fulfilled by highly asonicated DML dispersions. On the other hand, maximum at the phase transition temperature in a single parameter, the isothermal compressibility, is predicted on theoretical grounds (Phillips et al., 1975) and has been proposed to account for peaks at the T_m of other properties of lipid vesicles such as solute permeability (Papahadjopoulos et al., 1973; Linden et al., 1973), rate of insertion of exogenous lysophosphatides (Inoue and Kitagawa, 1974), and the number of 8-anilino-1-naphthalenesulfonate binding sites (Sackmann and Träuble, 1972). By the same reasoning, compressibility might also be expected to influence the rate of insertion of DML molecules into vesicles. This reasoning is fallacious, however, because it assumes constraints on the system that do not exist. Isothermal compressibility is a parameter that is critical for

the addition of components to a system only when that system has a fixed volume. Lipid vesicles are not rigidly bounded, so they may expand upon insertion of additional molecules with virtually no free energy change. Given this situation, the activation energy for separating the lipid molecules of the bilayer enough to insert a foreign molecule will depend less on isothermal compressibility than on forces of cohesion between bilayer lipid molecules and of adhesion between the foreign molecule and the aqueous phase.

There is a second reason for preferring an explanation of maximal growth that assumes a difference of two rate constants to one that invokes a change in a single rate constant dependent upon isothermal compressibility. In the case of transfer of DML molecules to DPL and mixed vesicles, which we believe occurs by a mechanism similar to that for DML vesicle exchange, the transfer rate does not diminish above the transition temperature of DPL. If isothermal compressibility were important, this rate should drop when the entire system enters the liquid-crystal state.

If a difference in rate constants governs the temperature maximum for lipid transfer, it appears likely that the relevant constants are those which describe the rate of dissociation of a DML molecule from a DML vesicle. If this step is limiting, but not as slow above as below the transition temperature, then the observed behavior is expected. This follows from the dependence of transition temperature on vesicle size; within a narrow range around the average transition temperature, the rate of loss of monomers from the small vesicles (melted) would exceed that from the large vesicles (unmelted) and thus the latter would receive monomers faster than they lost them. Above the temperature at which the large vesicles melt, they would lose their advantage and the transfer rate would again be low, although equilibrium would still favor large vesicles. (The alternative but less plausible possibility is that monomer insertion is rate limiting and that this step is faster for crystalline than for liquid–crystalline vesicles.) The transition end point is, as required by this mechanism, slightly higher (about 0.5°) after incubation. Such a mechanism is, however, only plausible until a method is developed to accurately measure transitions of the entire spectrum of vesicle sizes present in a given dispersion.

Growth of Ganglioside-Containing DML Vesicles. It is surprising that the kinetics of vesicle growth should change from first to second order upon the addition to DML vesicles of only a few percent gangliosides. Either vesicle fusion or lipid transfer upon collision is therefore indicated. It may be relevant to the former possibility, however, that lecithin vesicles (natural or synthetic above their phase transition temperature) containing lysolecithin will fuse with cell surface membranes (Martin and MacDonald, 1974) and that both lysophosphatides and gangliosides form spherical micelles when dispersed in aqueous solutions. The large area of the polar groups, relative to that of the acyl chains of these lipids, causes them to perturb lipid bilayers and perhaps, thereby, facilitates membrane fusion. With respect to phase transition parameters, purely DML and ganglioside-containing vesicles differ little (transition temperature and width change less than 1° upon incorporation of 3% ganglioside). This suggests that ganglioside-DML vesicles are only slightly perturbed, such that either a slight perturbation is sufficient to cause a significant change of behavior or that the gangliosides are segregated into highly unstable patches, leaving the bulk of the DML in a relatively pristine condition.

Prestegard and Fellmeth (1974) and Kantor and Prestegard (1975) have also investigated the growth of sonicated vesicles composed of DML and small amounts of another lipid. They found that the proton magnetic resonance intensity of the choline methyl group of vesicles composed of commercial DML decreased upon incubation. Since the spectral change they observed was opposite to that found when a coarse dispersion is sonicated, they interpreted the change seen upon incubation to mean that the average vesicle size increased. This interpretation was supported (Prestegard and Fellmeth, 1974) by results of gel permeation chromatography. Prestegard and Fellmeth found that growth rate exhibited a sharp maximum at around 19° and was dependent upon the presence of a contaminant (possibly myristic acid) in commercial DML. According to intensity vs. time plots, the process followed third-order kinetics. To the contrary, Kantor and Prestegard (1975) state that a tenfold variation in lipid concentration does not greatly affect initial rates and the process cannot therefore be third order. The lack of effect of lipid concentration implies zero-order kinetics, although Kantor and Prestegard drew no conclusion regarding the appropriate kinetic order, in spite of the fact that this parameter is critical to a determination of the mechanism of the process. Both Prestegard and Fellmeth (1974) and Kantor and Prestegard (1975) assert that DML vesicles become larger by fusion, apparently without considering alternative explanations consistent with their data. Given the questions regarding kinetics, the origin of the change upon sonication of the choline methyl absorption intensity (Lee et al., 1974), and ambiguities materializing when a phenomenon is strongly influenced by small amounts of impurities, little more can be said other than that our observations are similar to theirs with respect to the growth of vesicles and the existence of a temperature maximum thereof.

Redistribution of Lipids among Vesicles of Different Composition. Complete mixing of the lipid components of DML and DPL vesicles occurs fairly rapidly during incubation at temperatures at which both lipids are in the liquid-crystalline phase. The time course of the process indicates that lipid is transferred unidirectionally, DPL vesicles acquiring progressively larger numbers of DML molecules with time. It appears unlikely, then, that vesicle–vesicle fusion occurs. These data can be interpreted in terms of fusion only if DML vesicles are very much smaller than DPL vesicles. Electron microscopic evidence shows, however, that the two kinds of vesicles have similar size distributions, the average diameter in both cases being 50 ± 20 nm.

The present observations are most easily explained by a mechanism analogous to that suggested for DML vesicle growth; in this case, we focus not on the critical micellar concentration of DML for small as opposed to large vesicles, but on the critical micellar concentration of DML as opposed to that of DPL without regard to vesicle size. According to Smith and Tanford (1972), the free energy for transfer of DPL molecules from a nonpolar to an aqueous environment is 15.1 kcal/mol, the incremental free energy per methylene group being about 500 cal/mol. The corresponding free energy of transfer for DML molecules should then be about 13 kcal/mol. Given that DML and DPL vesicles are about the same size, a simple calculation shows that the DML critical micellar concentration is some 30 times greater than that of DPL. DML molecules will thus equilibrate with DPL and mixed DML–DPL vesicles. If DML molecules exhibit no preference between DPL and

mixed vesicles, then mixed vesicles will appear at the expense of DML vesicles, as we observed.

The increased rate of transfer of DML molecules to mixed vesicles may, as suggested above, reflect an increased rate of dissociation of molecules from vesicles that are above their transition temperature. This explanation is not entirely adequate, however, since the transfer rate increases even more at the transition temperature of the vesicles that are receiving DML molecules (DPL or mixed vesicles, depending upon how close the system is to equilibrium). In this situation, a potential additional influence may be a distribution of sizes of DPL and mixed vesicles. Temperatures near the transition temperature of these vesicles may encourage the elimination of the smallest thereof and promote the formation of larger and more stable vesicles which would lose DML more slowly. In any case, the persistence of a high transfer rate at temperatures above that of the phase transition of the receiving vesicles would be expected since the difference between the critical micelle concentrations of DML and DPL should persist whether or not these molecules are in crystalline or liquid-crystalline vesicles.

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