Calorimetric Detection of Curvature Strain in Phospholipid Bilayers

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ABSTRACT Phospholipids in biological membranes are arranged as bilayers. When constrained to pack into planar bilayers, certain phospholipids will form unstable structures as a consequence of their molecular shape and noncovalent bonding. This produces curvature strain which may provide energy for certain membrane processes. We demonstrate that an exothermic process associated with the relief of curvature strain can be detected calorimetrically. The enthalpy for the incorporation of a few percent lysophosphatidylcholine into large unilamellar vesicles of monomethyldioleoylphosphatidylethanolamine at pH 7.4 is exothermic but it is endothermic for stable bilayers such as this same lipid at pH 9 or dioleoylphosphatidylcholine at pH 7.4 or 9. The addition of lysophosphatidylcholine to monomethyldioleoylphosphatidylethanolamine at pH 7.4 is exothermic only for the addition of the first few percent of lysophosphatidylcholine and then it becomes endothermic. The size of the exothermic heat change is sensitive to changes in temperature, while the endothermic processes are relatively temperature-insensitive. The exothermic heat is also larger when 1 or 2 mol % of diolein is incorporated into vesicles of monomethyldioleoylphosphatidylethanolamine. These results are all consistent with the exothermic process corresponding to the relief of curvature strain in bilayers having a tendency to convert to the hexagonal phase. It provides a demonstration that considerable energy may be released upon the incorporation of certain molecules into membranes which have a low radius of spontaneous curvature.

INTRODUCTION

Phospholipids self associate in a variety of morphologically distinct modes including micelles, lamellar, hexagonal, and cubic phases. Lamellar phases will rearrange to other forms when the curvature strain of the individual monolayers of the bilayer becomes too large (Gruner, 1992). The spontaneous curvature of a monolayer can be either positive or negative, resulting in the formation of either "normal" phases with lipid headgroups oriented away from the lipid aggregate and toward the aqueous solvent or "inverted" phases in which the lipid headgroups are oriented toward the center of the lipid aggregate. When amphiphiles with spontaneous monolayer curvature of opposite sign are mixed, they can result in the formation of flat stable bilayers which have a large spontaneous radius of monolayer curvature.

Although phospholipids in biological membranes are believed to be arranged primarily as bilayers, a substantial fraction of the total phospholipid composition of these membranes is comprised by substances that will form inverted structures, such as the H_{II} phase, in purified form (Cullis and DeKruijff, 1979; Lindblom and Rilfors, 1989; Luzzati et al., 1993; Seddon, 1990). It is believed that the presence of lipids with such a property is important in modulating several functions of cell membranes (Epand, 1991; Gibson and Brown, 1993; Hein et al., 1992; Keller et al., 1993). These membrane functions may proceed with greater facility in the presence of nonbilayer-forming lipids, because these lipids increase the energy of the membrane and this energy can be released by coupling with another membrane process.

Lysophosphatidylcholine spontaneously forms micelles and therefore has an intrinsic spontaneous curvature which is opposite to that of phospholipids which form inverted phases. Lysophosphatidylcholine (Lichtenberg et al., 1991) and other detergents (Kresheck and Long, 1988) have been used to dissolve liposomes of different sizes to assess the contribution of bilayer curvature to the stability of these structures. The reaction of detergents with phospholipid vesicles is endothermic for all sizes of liposomes, for a process in which the liposome is completely dissolved by the detergent (Kresheck and Long, 1988; Lichtenberg et al., 1991). The fact that the process is endothermic can be understood, since the interactions between a single and a double chain amphiphile will not be as great as between two diacylphospholipids. In contrast to the micelle-forming lysophosphatidylcholine, *N*-methyl-1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (MeDOPE) forms inverted phase structures (Gruner et al., 1988; Siegel and Banschbach, 1990). MeDOPE has been used in a number of model system studies of membrane properties (Ellens et al., 1989; van Gorkom et al., 1992). When MeDOPE is constrained to remain in a lamellar phase each monolayer has a contribution to its latent energy from curvature strain. The higher the temperature, but at temperatures in which the lipid is still a bilayer, curvature energy is expected to increase. In the present work we measure heats of reaction between lysophosphatidylcholine and bilayer membranes. The relative changes in these heats for different lipids and for different conditions is considered in relation to curvature strain.

Received for publication 18 August 1993 and in final form 16 February 1994.

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0006-3495/94/05/1450/07 $2.00

EXPERIMENTAL PROCEDURES

Materials

Diacylphospholipids, 1,2-dioleoylglycerol (diolein) and lysophosphatidylcholine (L-monopalmitoylphosphatidylcholine) were purchased from

Avanti Polar Lipids, Alabaster, AL. The lipids showed a single spot on thin-layer chromatography (TLC) at a 50-μg load, using I_2 vapor for detection.

Preparation of large unilamellar extruded vesicles

Phospholipid was dissolved in chloroform/methanol (2:1, v/v), and diolein, if present, also added to this solution. The solvent was evaporated with a stream of nitrogen to deposit the lipid as a film on the wall of a test tube. Final traces of solvent were removed by placing the film in a vacuum desiccator for at least 2 h. The dried lipid films were hydrated with 20 mM 1,4-piperazinediethanesulfonic acid (Pipes), 0.15 M NaCl, 1 mM EDTA, 20 mg/liter NaN_3, adjusted to pH 7.4 (Pipes buffer). In the case of MeDOPE, the pH of the Pipes buffer was readjusted to 9.0 prior to being added to the lipid film or to pH 9.5 for the phosphatidylethanolamines. The lipid films were suspended into buffer by vigorous vortexing and then taken through five freeze/thaw cycles. The suspension was then extruded ten times through two polycarbonate filters having a pore size of 100 nm using a Lipex extruder (Vancouver, B.C.). In the cases of MeDOPE and the phosphatidylethanolamines, the vesicle suspensions were acidified to pH 7.4 prior to calorimetric measurement. The resulting large unilamellar extruded vesicles (LUVETS) were stored under argon and used the same day.

Size determination of LUVETS

The particle size distribution in the suspensions of LUVETS were determined by quasi-elastic light scattering. The analysis was performed using a Nicomp, Model 370 DLS Submicron Particle Sizer (Pacific Scientific Instruments, Silver Springs, MD). Particle size was determined from the calculated diffusion coefficient using the Stokes-Einstein equation. The particle size distribution was fit to a Gaussian curve, and the mean hydrodynamic particle diameter was calculated using software provided by the manufacturer.

Partitioning of lysophosphatidylcholine

The partitioning of lysophosphatidylcholine between the aqueous environment and the LUVETS was determined by separating the LUVETS by centrifugation followed by lipid analysis of the supernate fraction. To simulate the titration calorimetry, 0.8 or 4 mol % of lysophosphatidylcholine was added to LUVETS of MeDOPE or of dioleoylphosphatidylcholine. This corresponds to the lipid composition after one or after five injections of lysophosphatidylcholine. The LUVETS were then centrifuged at 350,000 *g* for 2 h at 25°C in a Beckman TL-100 centrifuge. Samples of the supernate were removed and lyophilized. The lipid in the dry residue was then taken up in chloroform/methanol (2:1, v/v) and applied to TLC plates together with pure lipid standards. The TLC plates were run with a mobile phase of chloroform/methanol/water (65/25/4, v/v). The dried plate was then exposed to iodine vapor, and the areas corresponding to the R_f of the two lipids were scrapped off; the lipid extracted with chloroform/methanol (2:1, v/v) and the solvent evaporated. Phosphate analysis was performed on the residue to determine the amount of lipid (Ames, 1966).

Titration calorimetry

Isothermal heats of reaction were measured using the Omega cell of a Microcal titration calorimeter (Wiseman et al., 1989). Solutions were degassed under vacuum prior to use. LUVETS (50 mM) were placed in the 1.3-ml reaction cell and thermally equilibrated prior to use. A solution of lysophosphatidylcholine (50 mM) was made in Pipes buffer at pH 7.4. The lysophosphatidylcholine was delivered into the reaction vessel in 10-μl aliquots from a 100-μl motor-driven syringe. Injections were made at 5-min intervals with continuous stirring at 400 rpm. The observed enthalpies were corrected for the small heat of dilution of lysophosphatidylcholine into buffer. The calorimeter was calibrated electrically. The data were analyzed using software provided by Microcal.

RESULTS

We titrated LUVETS of MeDOPE at 25°C or at 35°C with aliquots of lysophosphatidylcholine. At both temperatures we observe that an exothermic process occurs at low mole fractions of lysophosphatidylcholine, but that the enthalpy becomes positive after subsequent additions of lysophosphatidylcholine (Fig. 1). When the peaks become endothermic the shape appears more complex, suggesting that more than one process is involved; the nature of which can only be speculated on. The titration calorimetry runs presented in this paper were repeated on at least three separate occasions with similar results. Representative titrations are shown. The LUVETS of MeDOPE were made at pH 9 in order to obtain well hydrated liposomes but the titration presented in Fig. 1 was run after acidification to pH 7.4. Inclusion of a freeze/thaw cycle after acidification had no effect on the observed enthalpy. At pH 9, the MeDOPE is negatively charged and forms stable bilayers which do not convert to inverted phases. When titration calorimetry of MeDOPE is run at pH 9 without acidification, no exothermic reactions are observed but rather a series of endothermic reactions with each addition of lysophosphatidylcholine (not shown). Another lipid which would be expected to have little curvature strain is dioleoylphosphatidylcholine. The heat of mixing of lysophosphatidylcholine with LUVETS of dioleoylphosphatidylcholine is endothermic for all additions and compared with MeDOPE, it does not vary greatly with the mole fraction of lysophosphatidylcholine added (Fig. 2) nor with temperature (Fig. 3) or between pH 7.4 and 9 (data not shown). Control experiments with phosphatidylcholine vesicles at pH 9 followed by acidification or leaving the MeDOPE LUVETS for varying times and temperatures prior to titration demonstrated that none of the observed enthalpy was a consequence of titration of the more basic vesicle contents. In addition, performing the titration at pH 7.4 with LUVETS of dioleoylphosphatidylcholine made at either pH 9 or at pH 7.4 gave similar results (not shown). There is a small variation in the magnitude of the positive enthalpy upon addition of lysophosphatidylcholine to the dioleoylphosphatidylcholine. In some cases, such as the run presented at 25°C, the enthalpy increases slightly for the first three or four injections. Subsequently the enthalpy deceases with increasing injection number. Factors contributing to this variation are that the initial injection is sometimes lower because of diffusion of solute from the syringe during thermal equilibration and prior to the start of the series of injections. In addition, at very high injection number, when the ratio of lysophosphatidylcholine to diacylphospholipid is high, the enthalpy of reaction is expected to be zero. The decrease observed with increasing injection number may reflect, in part, an approach to this state. In addition there appear to be small variations which cannot be accounted for by these factors alone but their origin can only be speculated upon. For example, there may also be a contribution from curvature strain for the phosphatidylcholine. Nevertheless these variations are small compared with the large changes in both the magnitude and the sign of the enthalpy for the reaction with

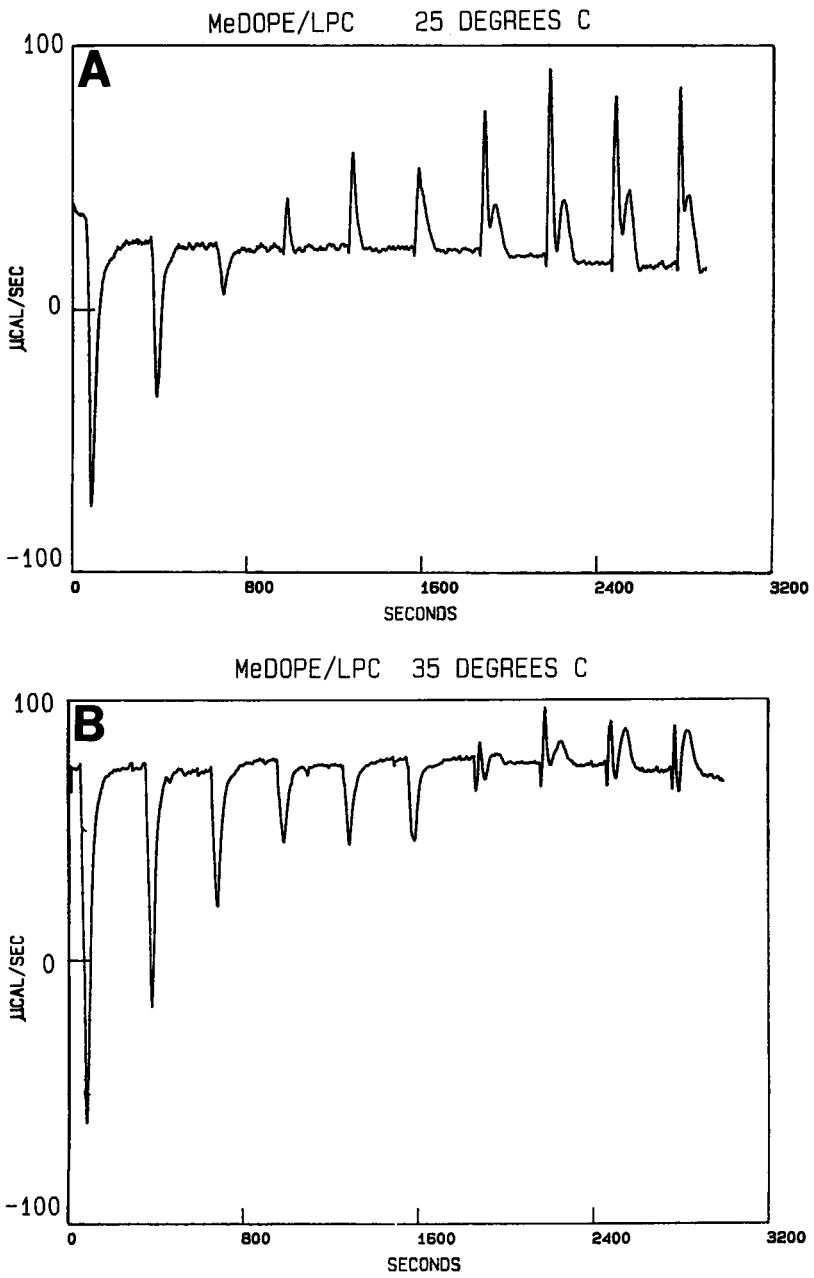

FIGURE 1 Titration calorimetry of lysophosphatidylcholine with MeDOPE. (*A*) Reaction at 25°C; (*B*) at 35°C. A 50 mM solution of MeDOPE (1.3 ml), in the form of LUVETS were titrated with 10 additions of 10 μl each of 50 mM lysophosphatidylcholine. Negative peaks represent exothermic reactions. The total span of the *y* axis is 200 μcal/s. The buffer is 20 mM Pipes, 1 mM EDTA, 150 mM NaCl, 0.02 mg/ml NaN_3, pH 7.4.

MeDOPE. The deviation from the observed average enthalpy is only about 20%. The enthalpy of reaction at high mole fractions of lysophosphatidylcholine with dioleoylphosphatidylcholine is similar to that observed with MeDOPE (Fig. 3).

Dynamic light scattering showed that the LUVETS of both MeDOPE as well as those of dioleoylphosphatidylcholine had average diameters of 100 nm which was not altered by the addition of 0.8 or 4 mole % of lysophosphatidylcholine. In addition, separation of the LUVETS by centrifugation demonstrated that less than 10% of the lipid remained in the supernate and that the composition of this lipid was essentially the same as the starting composition. This indicates that essentially all of the lysophosphatidylcholine partitions from a micellar solution into the LUVETS for both lipids. It would require high speeds and long times of centrifugation to quantitatively sediment phospholipid vesicles whose density is close to that of the solvent.

Addition of diolein decreases the stability of bilayers of MeDOPE (van Gorkom et al. (1992) and references therein). The initial exothermic reaction for the first injection of lysophosphatidylcholine into MeDOPE increases in magnitude from -1.6 ± 0.3 cal/mol MeDOPE to -1.8 ± 0.2 and -2.6 ± 0.6 cal/mol MeDOPE upon the addition of 1 or 2 mol % diolein, respectively. In addition to the magnitude of the enthalpy change for the initial injection increasing in the presence of diolein, the number of injections which gave exothermic peaks also increased in the presence of diolein. This is further analyzed in the discussion.

Phosphatidylethanolamines containing unsaturated acyl chains also form bilayers with curvature strain. Multilammelar vesicles of dioleoylphosphatidylethanolamine convert

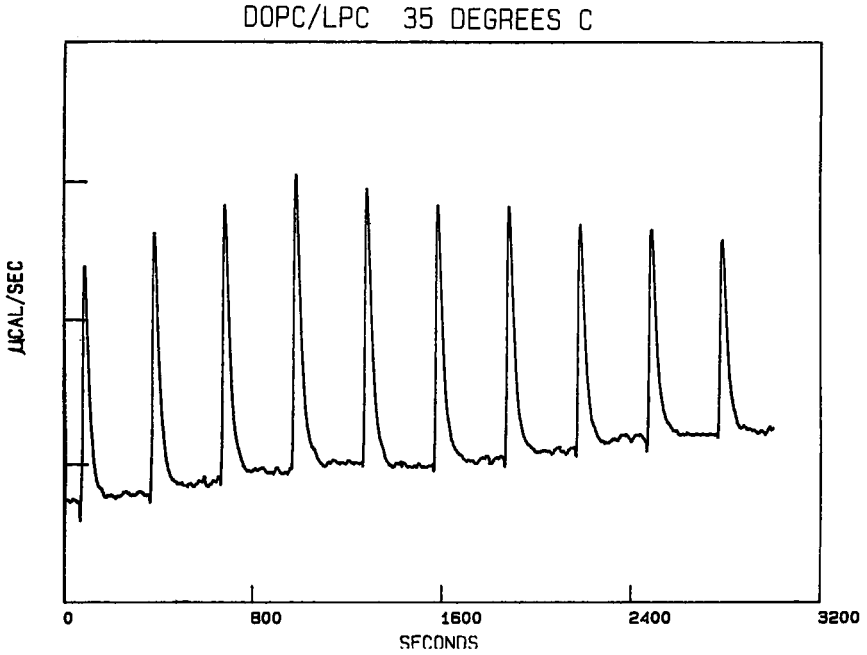

FIGURE 2 Titration calorimetry of lysophosphatidylcholine with dioleoylphosphatidylcholine. Reaction measured at 35°C with similar results given at 25°C. LUVETS were made in buffer at pH 7.4. Similar results were obtained using LUVETS made at pH 9 and acidified to pH 7.4. Other conditions as for Fig. 1. The tick marks on the ordinate represent 100 μcal/s.

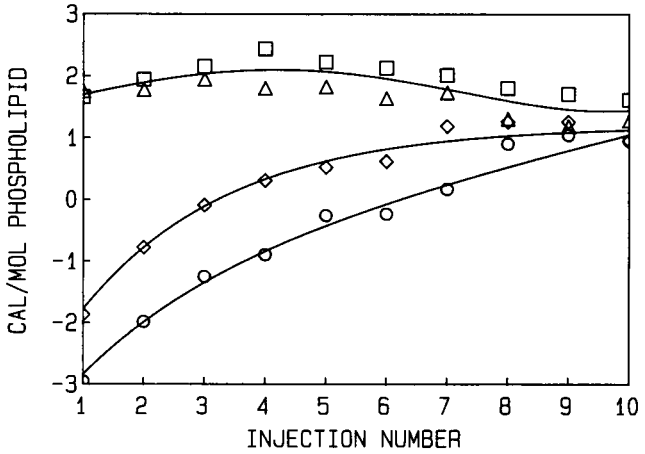

FIGURE 3 Calculated heats of reaction of lysophosphatidylcholine with MeDOPE or dioleoylphosphatidylcholine. Calculated values obtained from integration of peaks in titration calorimetry curves such as those shown in Figs. 1 and 2. The total observed heats for each injection were corrected for the heat of dilution of lysophosphatidylcholine. This heat of dilution represents a correction of only a few percent of the total observed enthalpy. Calculated enthalpy for each addition of 10 μl of lysophosphatidylcholine is given as cal/mol of dioleoylphospholipid. MeDOPE at 25°C (◇), MeDOPE at 35°C (○), dioleoylphosphatidylcholine at 25°C (□), and dioleoylphosphatidylcholine at 35°C (△).

from the L_α to the H_{II} phase at about 10°C. We attempted to make LUVETS from dipalmitoleoylphosphatidylethanolamine which has a transition from the L_α to H_{II} phase at 43°C. However when the LUVETS, made at pH 9.5, were acidified to pH 7.4 at the concentration required for the calorimetric measurements, the suspension was no longer a homogeneous dispersion, precluding calorimetric determination. We then made LUVETS using 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE). This lipid is not converted into the H_{II} phase until 71°C, however it exhibits an L_β to L_α transition at 25°C. In order to avoid contributions to the enthalpy of reaction from lysophosphatidylcholine-induced change in phase behavior, we performed the titration calorimetry with this lipid at 35°C. We observed that, as with MeDOPE, the enthalpy change becomes more endothermic with successive additions of lysophosphatidylcholine (Fig. 4). In contrast, 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) at this temperature becomes less endothermic with subsequent additions of the lysophosphatidylcholine (Fig. 4). While there are quantitative differences between the behavior of POPE and that of MeDOPE and their corresponding phosphatidylcholines, the behavior of the two systems ex-

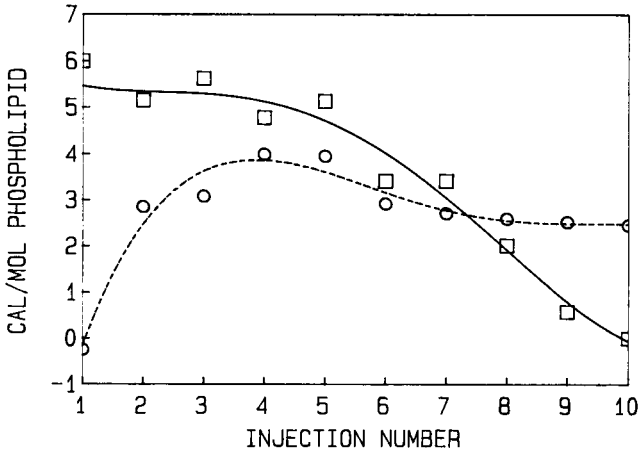

FIGURE 4 Calculated heats of reaction of lysophosphatidylcholine with POPE or POPC. Calculated values obtained by titration calorimetry. The total observed heats for each injection were corrected for the heat of dilution of lysophosphatidylcholine as described for Fig. 3. Calculated enthalpy for each addition of 10 μl of lysophosphatidylcholine is given as cal/mol of 1-palmitoyl-2-oleoylphospholipid. POPE at 35°C (○), POPC at 35°C (□).

hibit a number of striking similarities. In both cases the lipid with greater tendency to convert to the H_{II} phase exhibits a more exothermic reaction upon the first additions of lysophosphatidylcholine, while the phosphatidylcholine showed a more constant enthalpy of reaction for the first several additions of the lysophosphatidylcholine. The decrease in enthalpy at higher injection number was somewhat greater for POPC than for DOPC. In addition to differences in the nature of the acyl chains which might affect the enthalpy of mixing with lysophosphatidylcholine, there is also a difference in the structure of the headgroup between MeDOPE and POPE. One would expect hydrogen bonding among headgroups to be stronger in POPE than in MeDOPE. The disruption of these hydrogen bonds may be the cause for the higher enthalpy observed with POPE than with MeDOPE. We attempted to use lysophosphatidylethanolamine in place of lysophosphatidylcholine but found that the former lipid did not disperse in buffer and therefore could not be used.

DISCUSSION

The loss of curvature strain is the most likely explanation for the exothermic process observed with MeDOPE during the first injections of lysophosphatidylcholine. This is indicated by the observation that the exothermic process occurs with MeDOPE, a phospholipid which has a tendency to form inverted phases, but not with dioleoylphosphatidycholine which forms stable bilayers. Furthermore the magnitude of the exothermic process seen with MeDOPE is larger at higher temperatures. This is also as would be expected for curvature strain, since the diameter of H_{II} phase cylinders markedly decrease with increasing temperature as the entropic splay of the acyl chains increase (Tate and Gruner, 1987). In addition, the mixing of phospholipid bilayers with lysophosphatidylcholine is almost always found to be endothermic. The only exothermic reactions are with the initial addition(s) of lysophosphatidylcholine to membranes, such as those of MeDOPE or POPE, that have a tendency to convert to inverted phases. The heat of reaction with POPE is only slightly exothermic compared with MeDOPE, probably because a more stable hydrogen bonding network is broken with this lipid upon addition of lysophosphatidylcholine. Even with MeDOPE, the heat of reaction is endothermic at high pH or after several additions of lysophosphatidylcholine. This demonstrates that the cause of the exothermic process is different from simply the mixing of a single chain with a double chain lipid.

The partitioning of other nonpolar solutes into bilayers has been shown to be exothermic (Seelig and Ganz, 1991). This process has been termed the "nonclassical" hydrophobic effect and has recently been explained as arising from changes in the arrangement of the bilayer (Wimley and White, 1993). In our studies one would not expect a large hydrophobic effect, since the lysophosphatidylcholine is at a concentration above its critical micelle concentration (Lewis and Gottlieb, 1971) even after dilution into the reaction cell. Thus the lysophosphatidylcholine starts off in the syringe in micellar form with its hydrophobic acyl chains sequestered from water, and after addition to the LUVETS the detergent is incorporated into the bilayer, again with the acyl chains removed from contact with water. In addition, the present system differs in that the sign of the enthalpy changes upon subsequent addition of detergent and therefore the exothermic reaction is not an intrinsic characteristic of this system. Partitioning of substances into membranes can also result in a change of sign of the enthalpy if the reaction is performed near the phase transition temperature of the phospholipid (Kresheck and Nimsgern, 1983). This may be a consequence of the shift in the phase transition behavior of the system caused by the addition of the membrane additive (Epand and Sturtevant, 1981). This also is not applicable for the cases we have studied, since the phase transition temperatures of the lipids we have used are far removed from the temperature of measurement.

Although the sign of the enthalpy change for the first injections of lysophosphatidylcholine to MeDOPE is in accord with the suggestion that it arises from the relief of curvature strain, the magnitude of the curvature energy can only be estimated at this point. One of the difficulties is how to account for the endothermic contribution caused by the loss of noncovalent bonding interactions. A quantitative separation of this effect is even more difficult for the case of POPE and it will not be attempted. For MeDOPE, the enthalpy for the breaking of noncovalent bonds is likely to be represented by the positive enthalpy observed at high injection number with MeDOPE which is about the same as the enthalpy of reaction with dioleoylphosphatidylcholine. We therefore have subtracted the average enthalpy observed at high injection number from the observed enthalpy for each injection. This procedure leads to a value of zero curvature energy for dioleoylphosphatidylcholine, since there are only small deviations of the enthalpy from the average value. However, for the reaction of MeDOPE with lysophosphatidylcholine we obtain a total excess heat of reaction for all injections of -8.3 ± 1 cal/mol MeDOPE at 25°C and -12.3 ± 1 cal/mol at 35°C. A similar procedure, assuming the same plateaux value at high injection number (this plateaux is less well determined in the presence of diolein, since the change from exothermic to endothermic peaks occurs at higher injection number, with diolein present) leads to a calculated total excess heat of reaction at 25°C of -13 ± 1 cal/mol and -20 ± 3 cal/mol for MeDOPE with 1 or 2% of 1,2-dioleoylglycerol added, respectively. The calculated values of the total excess heat of reaction is not very accurate, since it is very dependent on the choice of the plateaux value which is subtracted from the enthalpy for each of the ten injections. Nevertheless the relative change in this value with increasing temperature or with the addition of 1,2-dioleoylglycerol are consistent with a large contribution of curvature strain to this excess negative heat of reaction.

The magnitude of the measured curvature strain enthalpic energy can be compared with free energy values which have been estimated from calculations based on experimental observations (Hui and Sen, 1989; Rand et al., 1990). Our

measurement is somewhat lower than these estimates but the theoretical values suffer from lack of accurate knowledge of the rigidity constant (Bo and Waugh, 1989) and the estimates from calorimetry are limited because of the factors discussed above as well as lack of knowledge about the sidedness of lysophosphatidylcholine in the LUVETS and its ability to undergo transbilayer diffusion.

The energy of reaction of lysophosphatidylcholine with phospholipid has been expressed per mole of phospholipid. The magnitude indicates that the excess negative enthalpy is smaller than the enthalpy of the H_{II} to L_{α} phase transition. This is to be expected as MeDOPE does not convert to the H_{II} phase until above 70°C. However it is possible that there are defects in the bilayer which are eliminated by lysophosphatidylcholine. Such defects may occur, for example, because the LUVETS are not perfectly spherical (Mui et al., 1993). The suggestion that membrane defects are being eliminated suffers from descriptive ambiguity, and we therefore favor the idea that curvature strain is being eliminated.

The analysis of the calorimetric results given above is in terms of the enthalpy per mole of phospholipid. The results, of course, can also be expressed per mole of lysophosphatidylcholine. The enthalpy of reaction of lysophosphatidylcholine with MeDOPE decreases with each successive injection. The enthalpy for the first injection into MeDOPE is -240 ± 30, -380 ± 35, and -480 ± 75 cal/mol lysophosphatidylcholine at 25, 35, 40°C, respectively. The value of the enthalpy of this single injection decreases with increasing injection number, however it does not represent the maximum value of this enthalpy change since using a smaller injection size the enthalpy per mole of lysophosphatidylcholine would likely be larger. There are, however, experimental limitations because of the smaller size of the observed heat evolution with addition of smaller aliquots of lysophosphatidylcholine. Thus the determination of the enthalpy per mole of lysophosphatidylcholine is limited by experimental constraints and the calculated values are lower limits. These constraints do not apply to the estimation of the enthalpy per mole of phospholipid, since this latter value is measured as a summation of all injections and is independent of injection size. The observed enthalpy of reaction of lysophosphatidylcholine with MeDOPE is the consequence of the opposing effects of the exothermic relief of curvature strain and the endothermic disruption of hydrocarbon packing and possibly also disruption of interactions among headgroups. In the case of dioleoylphosphatidylcholine, where the curvature energy is small, the observed enthalpy for the first injection of lysophosphatidylcholine was 234 ± 30 cal/mol lysophosphatidylcholine, independent of temperature between 25 and 45°C. This is close to the value, 330 cal/mol, obtained previously for the total solubilization of 1-palmitoyl-2-oleoylphosphatidylcholine with lysophosphatidylcholine (Lichtenberg et al., 1991). Thus for a hypothetical substance which did not disrupt noncovalent bonding among phospholipids the enthalpy of insertion into a bilayer with curvature strain would be even more exothermic by about 234 cal/mol. In either case, a considerable amount of heat is evolved per mole of bilayer stabilizer when such molecules are inserted into membranes which are under a high curvature strain. If this energy were coupled to a membrane event, such as the conformational change in a protein, it could be the driving force responsible for such processes.

We are grateful to Dr. Sol Gruner for helpful discussions.

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