*Biochimica et Biophysica Acta*, 448 (1976) 245–264


BBA 77450

# STUDIES ON MEMBRANE FUSION

## 1. INTERACTIONS OF PURE PHOSPHOLIPID MEMBRANES AND THE EFFECT OF MYRISTIC ACID, LYSOLECITHIN, PROTEINS AND DIMETHYLSULFOXIDE

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(Received March 22nd, 1976)

SUMMARY

The interaction and mixing of membrane components in sonicated unilamellar vesicles and also non-sonicated multilamellar vesicles prepared from highly purified phospholipids suspended in NaCl solutions has been examined. Electron microscopy and differential scanning calorimetry were used to characterize the extent and kinetics of mixing of membrane components between different vesicle populations. No appreciable fusion was detected between populations of non-sonicated phospholipid vesicles incubated in aqueous salt (NaCl) solutions. Mixing of vesicle membrane components via diffusion of phospholipid molecules between vesicles was observed in populations of negatively charged phosphatidylglycerol vesicles but similar exchange diffusion was not detected in populations of neutral phosphatidylcholine vesicles. Incubation of sonicated vesicle populations at temperatures close to or above the phospholipid transition temperature resulted in an increase in vesicle size and mixing of vesicle membrane components as determined by a gradual change in the thermotropic properties of the mixed vesicle population. The interaction of purified phospholipid vesicles was also examined in the presence of myristic acid and lysolecithin. Our results indicate that while these agents enhance mixing of vesicle membrane components, in most cases mixing probably proceeds via diffusion of phospholipid molecules rather than by fusion of entire vesicles. Increased mixing of vesicle membrane components was also produced when vesicles were prepared containing a purified hydrophobic protein (myelin proteolipid apoprotein) or were incubated in the presence of dimethylsulfoxide. In these two systems, however, the evidence suggests that mixing of membrane components results from the fusion of entire vesicles.

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Abbreviation: TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethane-sulfonic acid.

## INTRODUCTION

Although membrane fusion is important for a wide variety of cellular and subcellular activities (for reviews see refs. 1–4) very little is known concerning the mechanism at the molecular level. The difficulties in obtaining information on the molecular events associated with membrane fusion in whole cell systems have prompted the search for simplified model systems. Phospholipid vesicles [5, 6] appear to be a convenient and useful system for such studies and have been used recently in several laboratories for this purpose [7–15]. However, much of the published work claiming to have demonstrated fusion in these systems has not provided definitive evidence that fusion has taken place. For example, reliance on single criteria such as increases in vesicle size [9] or the transfer of spin labels between vesicles [7, 11] and changes in NMR spectra [9, 10, 13] cannot differentiate between mixing of vesicle membrane components caused by diffusion of individual molecules and mixing resulting from true fusion of entire vesicles. Consequently, there is considerable ambiguity concerning the molecular mechanism underlying data purporting to show vesicle fusion using these techniques. Interpretation of other published data claiming to show fusion between vesicles is complicated by the use of impure phospholipids [9], which can result in a large variation in the kinetics of the spectral changes used to monitor exchange of material between vesicles. The relative instability of vesicles that have not been "annealed" has been discussed elsewhere [13].

A further complicating factor in most of the above studies is the exclusive use of sonicated phospholipid vesicles. Such vesicles have been shown to be "strained" [16] probably because of the small radius of curvature which requires different numbers of molecules on the inner and outer monolayers [17]. Although sonicated unilamellar vesicles are used extensively and are believed to be stable structures, it is of importance to establish whether membrane interactions between such vesicles, involving either fusion or molecular diffusion, result simply from their relatively "strained" configuration or whether such interactions reflect inherent characteristics of lipid bilayer membranes.

In this paper we present data on the interaction and mixing of membrane components in both sonicated unilamellar vesicles and non-sonicated multilamellar vesicles prepared from four different highly purified phospholipids suspended in NaCl solutions. We have used electron microscopy to monitor changes in vesicle size and differential scanning calorimetry to monitor the extent and the kinetics of mixing of membrane components between different populations of vesicles.

In addition, we also present results on the effect of common lipid contaminants such as free fatty acids and lysolecithin, a lipid-soluble (hydrophobic) protein, and dimethylsulfoxide on the rate of mixing of vesicle membrane components. The results indicate that the mixing of membrane components observed in several systems with phospholipid vesicles does not involve true fusion of vesicles but is probably due to molecular diffusion. This conclusion is in general agreement with another recent study [15]. However, true fusion of pure phospholipid vesicle membranes can be induced by $Ca^{2+}$ [8], and this is discussed in detail in the next two papers in this series.

## MATERIALS AND METHODS

*Lipids.* Phospholipids were synthesized in this laboratory [18, 19] and contained no detectable impurities as determined by thin-layer chromatography on silica gel H and a solvent of chloroform/methanol/7M ammonia (230: 90: 15, v/v). Dipalmitoyl phosphatidylcholine (1,2-dihexadecyl-*sn*-glycerol-3-phosphorylcholine) and dimyristoylphosphatidylcholine (1,2-ditetradecyl-*sn*-glycero-3-phosphorylcholine) were synthesized according to Robles and Van Den Berg [20]. Dipalmitoyl phosphatidylglycerol and dimyristoyl phosphatidylglycerol were synthesized as before [18]. Lysolecithin was obtained from Applied Science Labs (State College, Pa.) myristic acid from Fluka, AG (Buchs, Switzerland, puriss, > 99 %) and stearylamine from K and K Laboratories.

*Preparation of vesicles.* Phospholipid vesicles (liposomes) were prepared in NaCl buffer solution as described previously [21, 22], either by mechanical vortex shaking or by sonication (where stated) for 1 h in a bath-type sonicator [22]. The temperature during shaking, sonication (where stated) and subsequent equilibration (1 h) was 2–5 °C above the transition temperature ($T_c$) of the particular lipid preparation. Unless stated otherwise, the buffer solution contained 100 mM NaCl, 2 mM *N*-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid (TES), 2 mM L-histidine and 0.1 mM EDTA, at pH 7.4. The lipid concentrations used in different experiments are stated in the figure legends.

*Differential scanning calorimetry.* Differential scanning calorimetry was performed as before [19] with a Perkin-Elmer DSC-2, at sensitivity range 1 mCal/s. and heating rate of 5 °C per min.

*Electron microscopy.* Negative staining electron microscopy was performed using 2 % ammonium molybdate which was added either after or during the preparation and sonication of vesicles with similar results. The buffer used for these experiments contained 100 mM ammonium acetate instead of NaCl [23]. Freeze fracture was performed as before [24].

*Other chemicals.* The major proteolipid apoprotein prepared from human brain myelin [25] generously provided by Dr. M. Moscarello, was mixed with the lipids in chloroform/methanol/water as described before [25]. L-Histidine and TES were purchased from Sigma, dimethylsulfoxide from Eastman Kodak, Rochester, N.Y., and silicic acid from Mallinckrodt (AR, 100 mesh). Water was twice distilled, the second time in an all glass apparatus.

## RESULTS AND DISCUSSION

### *Interaction between sonicated vesicles of pure phospholipids*

Several recent publications have claimed to have demonstrated fusion of sonicated phosphatidylcholine vesicles [7, 9]. However, our previous studies [8] had indicated that little if any fusion occurred between populations of non-sonicated phosphatidylcholine vesicles at temperatures above their $T_c$. It was therefore considered of interest to re-examine the problem in detail applying our techniques to sonicated vesicle populations.

Fig. 1 shows the calorimetric properties of vesicles prepared from dipalmitoyl phosphatidylcholine (Fig. 1B) and dipalmitoyl phosphatidylglycerol (Fig. 1A). In

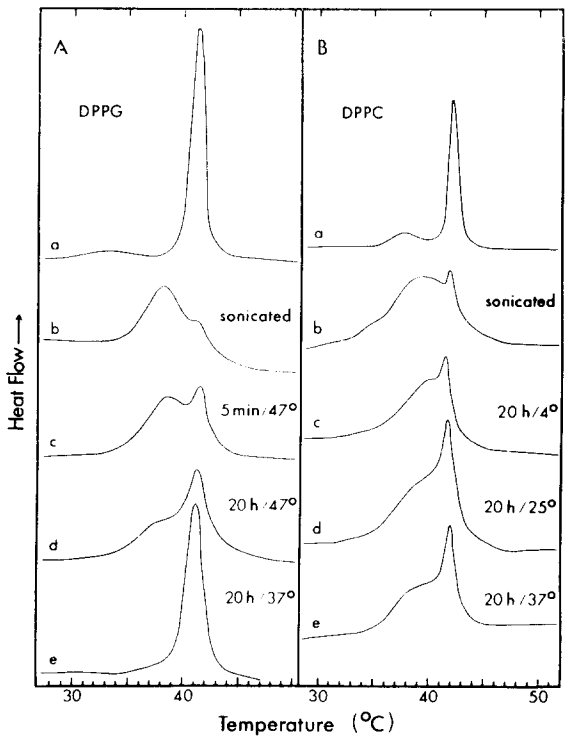


Fig. 1. Effect of sonication and subsequent incubation on the thermotropic properties of pure phospholipid vesicles. A: dipalmitoyl phosphatidylglycerol (DPPG) dispersed at 45 °C in 10 mM NaCl, 2 mM histidine, 2 mM TES, 0.1 mM EDTA, pH 7.4, at concentration 8 $\mu$mol/ml; (a) non-sonicated; (b) after sonication (at 45 °C) and ultrafiltration (at 22 °C). The remaining samples were prepared as in (b) and were then incubated further under the following conditions: (c) 5 min at 47 °C; (d) 20 h at 47 °C; (e) 20 h at 37 °C. B: dipalmitoyl phosphatidylcholine (DPPC) dispersed in 100 mM NaCl buffer, pH 7.4 at concentration 20 $\mu$mol/ml; (a) non-sonicated; (b) after sonication (at 45 °C) and ultrafiltration (at 22 °C). The remaining samples were prepared as in (b) and were then incubated as follows: (c) 4 °C for 20 h; (d) 25 °C for 20 h; (e) 37 °C for 20 h.

both cases the preparation was sonicated at 45 °C and then ultrafiltrated by pressure dialysis at room temperature. Curves b in Fig. 1 were obtained after sonication and concentration of vesicles by ultrafiltration and show that sonicated vesicles have a much broader transition temperature (from 3 to 17 °C range) and a mid-point approximately 2 °C lower than the non-sonicated vesicle samples* shown in

* In a recent paper DeKruyff et al. [39] have reported that sonication of phosphatidylcholine did not produce any changes in the thermotropic properties as studied by differential scanning calorimetry. The difference could be for several reasons, including incomplete sonication (in case of no change) or formation of degradation products (in case of lowering and broadening of the $T_c$). Since the effects in our case are reversible with time and the sonication is milder than that used by DeKruyff et al. (bath type sonication avoids direct contact with the probe) we assume that the changes in $T_c$ are not due to chemical degradation during sonication, but rather due to the strain imposed by the high curvature of the unilamellar vesicles [16]. The discrepancy could also be related to differences in lecithin preparation (DeKruyff et al. did not observe with unsonicated lecithin the well-documented premelt transition) or differences in medium composition (DeKruyff et al. used 25 mM Tris acetate

curves a. In both cases there was a residual small peak at the same temperature as the main transition of the non-sonicated material. The pre-melt transition also seems to have disappeared in both cases.

Incubation of the sonicated samples of dipalmitoyl phosphatidylcholine (Fig. 1B) for 20 h at 4 °C (curve c), at 25 °C (curve d) or at 37 °C (curve e) revealed a change in the thermogram with increasing heat associated with the transition at 42.5 °C compared to the broader transition at 39.2 °C, suggesting the formation of larger vesicles. This effect was more pronounced at higher temperatures. Similar and more clear-cut results were obtained with sonicated dipalmitoyl phosphatidylglycerol vesicles (Fig. 1A). In this case, incubation at 47 °C for 5 min produced an appreciable shift toward the 41.2 °C transition (curve c), which was much more pronounced after 20 h incubation at this temperature (curve d). Prolonged incubation (20 h) at 37 °C produced a quantitative shift to 41.2 °C transition (curve e).

The shift in the transition to a higher temperature found in the sonicated vesicles could be interpreted as indicating that fusion of sonicated vesicles has occurred to produce larger vesicles. This interpretation would be in accord with other recent observations on sonicated vesicles in which broadening of NMR spectra accompanying the incubation of sonicated vesicles was interpreted as indicating that fusion was taking place [9, 10]. However, as we shall show later in this paper, the phenomenon can be explained by an alternative mechanism that does not involve fusion.

In order to relate the above calorimetric data to possible changes in vesicle size, electron microscopic measurements were made on replicate vesicle preparations. Vesicles were prepared and sonicated as described above, except for the ultrafiltration step which was omitted since it was not needed. Dipalmitoyl phosphatidylglycerol vesicles were mixed at room temperature with the stain (ammonium molybdate) and dried directly on the grid either immediately (within 1 h) or after incubation for 20 h at 6 or at 45 °C. Measurements of the number of individual vesicles with diameters within specified length limits, revealed that incubation of sonicated vesicles at 45 °C produced populations with diameters above 3000 Å, (Fig. 2). This was similar to the non-sonicated vesicles and drastically different to the original vesicle populations present within 1 h after sonication, and the vesicles incubated for 20 h at 6 °C (Fig. 2). Similar but less pronounced changes were found during incubation of dipalmitoyl phosphatidylcholine vesicles (results not shown). It was not possible to ascertain, however, whether the large vesicles obtained after incubation of sonicated preparations resembled non-sonicated vesicles in being multilamellar since the stain did not penetrate within the vesicles [23] and the interior lamellae could not be seen.

The above results indicate that incubation of sonicated phospholipid vesicles at temperatures close to the main enthothermic transition results in vesicles of a larger size. This process reverses the effect of sonication (which produces a lower $T_c$) and restores the $T_c$ to that of non-sonicated vesicles. These results were more pronounced with negatively charged dipalmitoyl phosphatidylglycerol vesicles compared with neutral dipalmitoyl phosphatidylcholine vesicles. This is the opposite of what

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in $^2H_2O$ rather than 100 mM NaCl in $H_2O$). Reversible lowering of the $T_c$ following sonication of lecithin similar to that found here has also been observed in a more recent paper [40] and also earlier [19].

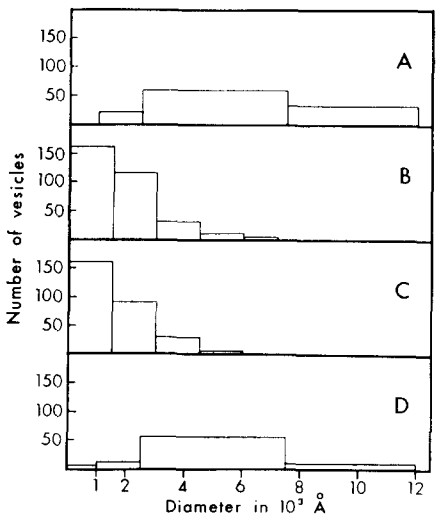


Fig. 2. Effect of sonication and subsequent incubation at different temperatures on the size of pure phospholipid vesicles as determined by negative stain electron microscopy. Dipalmitoyl phosphatidylglycerol was dispersed in 100 mM NaCl buffer, pH 7.4 at concentration 8 μmol/ml, sonicated for 1 h at 45 °C, and mixed with 2 % ammonium molybdate at room temperature just before drying on the grid as described in Materials and Methods: (A) before sonication; (B) immediately after sonication; (C) sonicated and incubated at 6 °C for 20 h; (D) sonicated and incubated at 45 °C for 20 h.

would be expected from collision-limited fusion events. We therefore undertook further experiments to ascertain whether the observed size changes resulted from the fusion of vesicles or from other phenomena such as the exchange of free molecules between vesicles by diffusion.

Detailed freeze-fracture electron microscopic observations on the interaction of sonicated vesicles of differing composition will be presented elsewhere. It is of interest to note, however, that sonicated dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol vesicles do not display the characteristic spherical shape seen with sonicated preparations of dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine vesicles (approximate diameter 300 Å) and instead appear as larger flattened ellipsoids (approximate diameter 1000 Å). Further, a more detailed comparison of vesicle size obtained by negative stain and freeze-fracture electron microscopy with these phospholipids is needed in order to explain the large variation in size shown in Fig. 2B.

*Interactions between non-sonicated vesicles or pure phospholipids*

In a previous publication [8] we reported on the interaction of multilamellar dipalmitoyl phosphatidylcholine vesicles with multilamellar distearoyl phosphatidylcholine vesicles, performed at temperatures above their respective $T_c$ and then incubated at 58 °C for up to 20 h. Mixing of membrane components from the two populations of vesicles was monitored by differential scanning calorimetry. The main endothermic peak of each of the above membranes is well-defined: 42 °C for dipalmitoyl phosphatidylcholine, 56 °C for distearoyl phosphatidylcholine and 51 °C for an equimolar mixture of the two. It was observed that even prolonged incubation

(20 h at high temperatures, 58 °C) did not change significantly the calorimetric spectrum of the individual components, indicating little if any mixing of molecules between the two populations of membranes [8].

We have recently repeated this experiment using pre-formed multilamellar vesicles of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles incubated together at 45 °C for 20 h, at a concentration of 5 μmol per ml for each lipid. The results indicated no significant mixing since there was no detectable change in the calorimetric characteristics of the individual components (results not shown). We conclude from this result, as well as our previous data [8], that no significant degree of fusion occurs between neutral lecithin membranes, and also that no significant exchange of molecules occurs from one membrane to another.

A recent publication has implicated a negatively charged impurity as

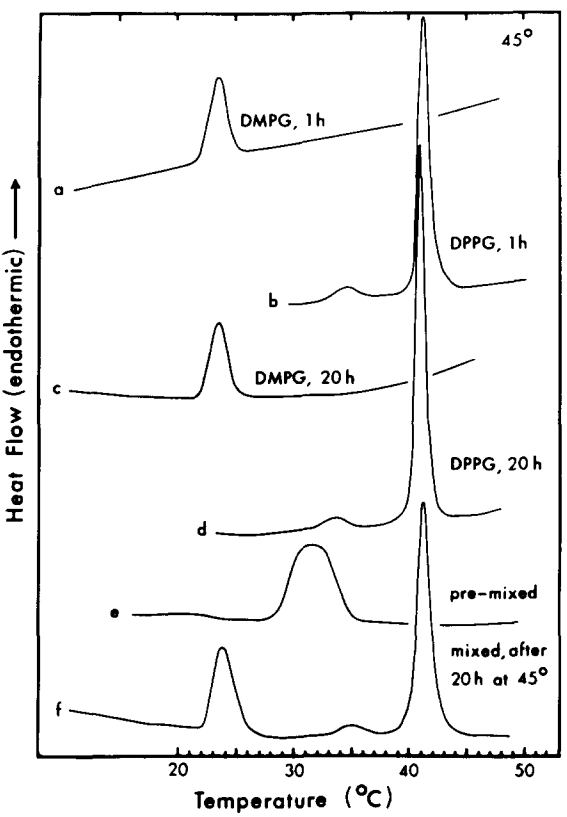


Fig. 3. Differential scanning calorimetry of pure and mixed phosphatidylglycerol vesicles. In all cases the phospholipids were suspended by shaking in 100 mM NaCl buffer, pH 7.4, at 45 °C at a concentration of 5 μmol/ml. They were then incubated further at 45 °C for the indicated times: (a) dimyristoyl phosphatidylglycerol (DMPG), 1 h; (b) dipalmitoyl phosphatidylglycerol (DPPG), 1 h; (c) dimyristoyl phosphatidylglycerol, 20 h; (d) dipalmitoyl phosphatidylglycerol, 20 h; (e) mixture of dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol suspended together and incubated for 1 h at 45 °C; (f) vesicles of dimyristoyl phosphatidylglycerol and vesicles of dipalmitoyl phosphatidylglycerol as in (a) and (d) above were mixed after incubation for 20 h at 45 °C and analyzed immediately.

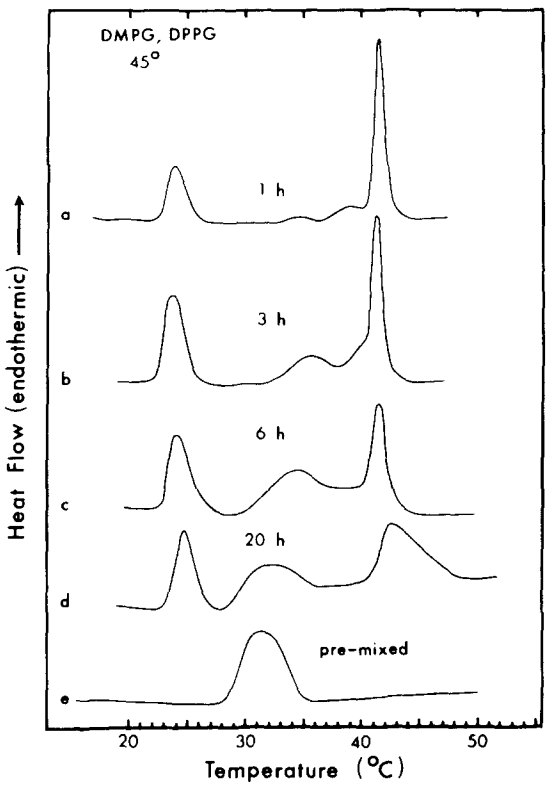


Fig. 4. Molecular mixing in pre-formed vesicles of dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol detected by differential scanning calorimetry. The vesicles were prepared as described in Fig. 3(a) and (b) and incubated together at 45 °C for the following times: (a) 1 h; (b) 3 h; (c) 6 h; (d) 20 h; (e) vesicles formed from an equimolar mixture of the two lipids.

promoting the fusion of vesicles [10]. We therefore examined the role of negative surface charge on the molecular mixing and fusion between non-sonicated lipid vesicles. Two populations of vesicles composed of pure acidic phospholipids with different melting points were chosen for this series of experiments: dimyristoyl phosphatidylglycerol with a $T_c$ at approximately 24 °C (Fig. 3, curve a); and dipalmitoyl phosphatidylglycerol with a $T_c$ at approximately 41 °C (Fig. 3, curve b). These vesicles were shown to be stable when incubated at 45 °C for up to 20 h (Fig. 3, curves c and d) in 100 mM NaCl buffer, pH 7.4 and no changes in the calorimetric characteristics were found. If the two phospholipids were premixed in chloroform and suspended together, they then exhibited a single thermotropic transition at approximately 31 °C (curve c). If the individual suspensions were mixed after 20 h incubation at 45 °C the individual components were not altered (curve f).

Incubation of the two populations of vesicles together at 45 °C produced changes in their thermotropic characteristics (Fig. 4). Examination of curve d which was obtained following incubation of the two vesicle populations together for 20 h at 45 °C, indicates the presence of three endothermic transitions. Two of these are similar (but broader) to those of the individual components while the third at 32 °C

is close to that of the pre-mixed lipid suspension (31.5 °C; curve e). The formation of an equimolar mixture following prolonged incubation of the vesicles, together with the persistence of some of the original components could be interpreted as indicating that fusion between the two populations of vesicles had occurred. However, the kinetics of the reaction are not consistent with this interpretation. Careful examination of the changes occurring at different times indicates that a new peak appears after 1 h incubation at 39 °C (curve a). This peak is augmented in size (relative area under the peak compared with the initial components) but after 3 h now has a midpoint at 36 °C and undergoes a further shift to 34.5 °C after 6 h and finally to 32 °C after 20 h.

Electron microscopic examination of the two populations of vesicles, immediately after their formation reveals a roughly similar range of sizes. It would therefore be expected that fusion between vesicles of dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol would produce equimolar mixtures having a $T_c$ at 31 °C. The shift of the third peak from temperatures close to the $T_c$

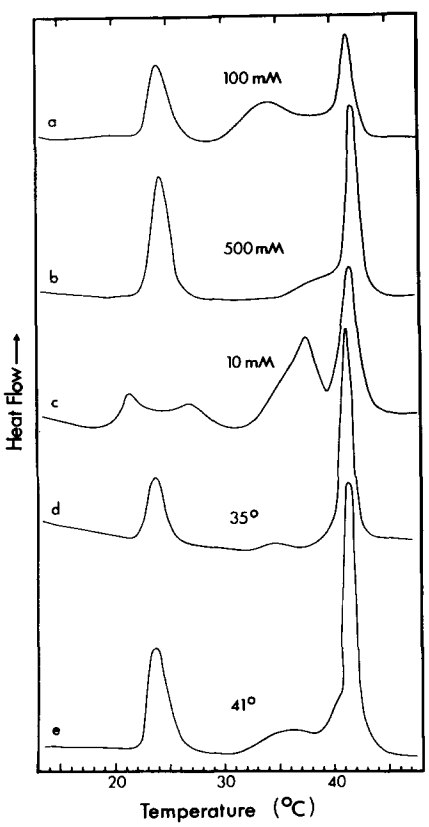


Fig. 5. Molecular mixing with pre-formed vesicles of dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol detected by differential scanning calorimetry. Vesicles were formed as in Fig. 3(a) and (b), except as indicated below, and incubated together for 6 h: (a) vesicles formed and incubated in 100 mM NaCl buffer at 45 °C; (b) as in (a) except 500 mM NaCl; (c) as in (a) except 10 mM NaCl; (d) incubated at 35 °C in 100 mM NaCl; (e) incubated at 41 °C in 100 mM NaCl.

of dipalmitoyl phosphatidylglycerol to lower temperatures closer to those of the equimolar mixture indicates that continuous enrichment of the dipalmitoyl phosphatidylglycerol membranes with increasing amounts of dimyristoyl phosphatidylglycerol molecules is occurring. It is thus reasonable to consider that the changes observed during the incubation of phosphatidylglycerol membranes are in fact due to diffusion of dimyristoyl phosphatidylglycerol molecules to the dipalmitoyl phosphatidylglycerol membranes rather than fusion. This possibility is strengthened by experiments studying the effect of ionic strength on the reaction between dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol membranes (Fig. 5). As shown by the differences between curves a, b and c, increasing the monovalent salt concentration to 500 mM inhibited the reaction (curve b) while decreasing to 10 mM produced two intermediate peaks indicating considerable intermixing.

It would be expected that a collision-dependent fusion reaction between charged vesicles would be enhanced by increasing salt concentration and reduced by decreasing it. It would also be expected (by extrapolation of effects of salts on the critical micellar concentration of negatively charged surfactants) that increasing salt concentration would diminish and decreasing salt concentration would enhance solubility of the monomeric species. The results in Fig. 5a thus suggest that vesicles of this type interact and undergo mixing through diffusion of free molecules of the most soluble component (dimyristoyl phosphatidylglycerol) to the other membranes rather than through fusion of the vesicle membranes. The effect of lowering the temperature (compare curves a, d and e) at the same ionic strength is also shown in Fig. 5. Mixing was significantly reduced by lowering the temperature from 45 °C (Curve a) to 41 °C (Curve e) and completely inhibited at 35 °C (Curve d). This indicates that both membranes must be above their $T_c$ before any mixing can occur.

*Effect of myristic acid on the interaction phospholipid vesicles*

In a previous publication [8] we studied the interaction of two populations of non-sonicated phosphatidylcholine vesicles, one containing a long chain anion (20 % palmitoleic acid in dipalmitoyl phosphatidylcholine; $T_c$ = 39 °C) and the other a long chain cation (20 % stearyl amine in distearyl phosphatidylcholine; $T_c$ = 58 °C). Although immediate flocculation and precipitation was observed following incubation of the two vesicle populations together (presumably due to charge interactions) no evidence of fusion was detected after 2 h incubation at 60 °C. This conclusion was based on the absence of a third intermediate peak in thermograms similar to those shown in Figs. 4 and 5. The evidence did indicate, however, that a considerable amount of molecular mixing between the two populations of vesicles was occurring since the two initial endothermic peaks shifted progressively toward each other to intermediate temperatures [8], indicating that exchange diffusion of molecules between the oppositely charged membranes was occurring without complete fusion.

Prestegard and colleagues [9, 10] have studied the interaction between sonicated phosphatidylcholine vesicles using NMR and claimed to have demonstrated fusion between phosphatidylcholine vesicles. However, the changes in the NMR spectra following incubation of vesicles observed by these investigators merely indicate an increase in vesicle size. In a subsequent paper, Kantor and Prestegard [10] reported data indicating that their earlier observations were due to the presence of an impurity in the phosphatidylcholine vesicles. The impurity was characterized

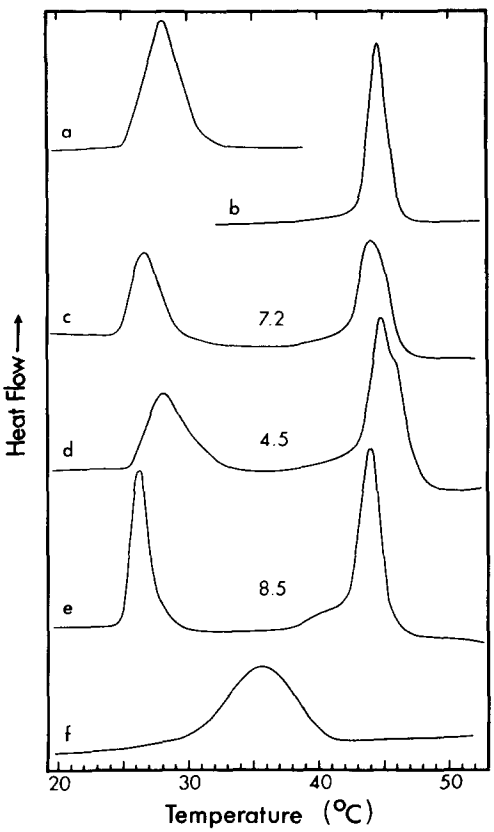


Fig. 6. Effect of myristic acid on the thermotropic properties and molecular mixing in non-sonicated phosphatidylcholine vesicles. In all cases vesicles were formed by mixing 20 $\mu$mol of phosphatidylcholine with 2 $\mu$mol of myristic acid in chloroform, followed by evaporation and suspension in 3.5 ml of 100 mM NaCl buffer, pH 7.2, at 50 °C for 1 h. Aliquots were then taken and incubated either together or separately at 50 °C for 4 h as indicated below: (a) 10 % myristic acid in dimyristoyl phosphatidylcholine; (b) 10 % myristic acid in dipalmitoyl phosphatidylcholine; (c) equimolar mixture of (a) and (b) incubated at pH 7.2; (d) as in (c) except at pH 4.5; (e) as in (c) except at pH 8.5; (f) 10 % myristic acid in equimolar mixture of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine suspended together in 100 mM NaCl buffer, pH 7.2.

as myristic acid and it was suggested that this agent was able to promote fusion of lecithin vesicles. In view of this ambiguity concerning the capacity of pure lecithin membranes to fuse and the role of fatty acids in promoting fusion of such membranes we examined the effect of myristic acid on the interaction of pure phosphatidylcholine membranes in our system. Fig. 6 describes the results obtained with two populations of vesicles composed of dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine each containing 10 % mol myristic acid. They were each formed separately at temperatures above their $T_c$, and then incubated together for 4 and 20 h at 50 °C, at different pH values. As shown in Fig. 6 although some broadening and movement of the peaks toward each other was found, no appreciable accumulation of material melting at the temperature of the complete mixture was obtained. Similar results were obtained at pH 7.2 (curve c), pH 4.5 (curve d) and pH 8.5 (curve e), and

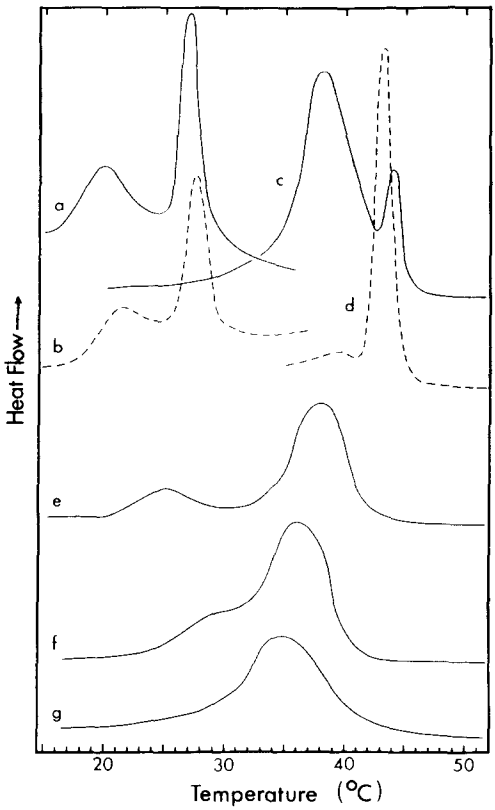


Fig. 7. Effect of myristic acid on the thermotropic properties and molecular mixing in sonicated phosphatidylcholine vesicles. In all cases vesicles were made by mixing 120 $\mu$mol of phosphatidylcholine with 12 $\mu$mol of myristic acid in chloroform, evaporated, and suspended by shaking in 1 ml of 100 mM NaCl buffer, pH 7.2 at 50 °C, for 1 h. The suspension was then centrifuged briefly, 0.7 ml removed from the supernatant, and the pellet resuspended and sonicated at 50 °C for 1 h: (a) 10 % myristic acid in dimyristoyl phosphatidylcholine after sonication and incubation at 50 °C for 3 h; (b) as in (a) except incubation at 50 °C for 20 h; (c) 10 % myristic acid in dipalmitoyl phosphatidylcholine after sonication and incubation at 50 °C for 3 h; (d) as in (c) except incubation at 50 °C for 20 h; (e) aliquots from (a) and (c) mixed immediately following sonication and incubated at 50 °C for 3 h; (f) same as in (e) except for incubation at 50 °C for 20 h; (g) a mixture of all the components in chloroform, dispersed and sonicated together and incubated at 50 °C for 1 h.

in the presence of 2 mM $CaCl_2$ (not shown). Similarly, incubation at lower temperatures failed to produce any evidence of substantial mixing and good preservation of the original two peaks was observed (results not shown).

In order to approximate our conditions to those employed by Kantor and Prestegard [10] we examined the interaction of the above vesicles after sonication. The results are shown in Fig. 7. It is apparent from the data in curves a and c that sonication produces a complex thermotropic behavior with two main endothermic peaks. This is probably due to incomplete sonication because of the very high concentrations of lipid employed. In any case the lower peak which is 5–7 °C lower than the other (which corresponds to the non-sonicated material) is diminished with time following incubation of the vesicles separately at 50 °C for 20 h (curves b, d). Mixing

and incubation of the two vesicle populations at 50 °C for 3 h (pH 7.2) results in two main peaks at intermediate temperatures, 27 and 38 °C (curve e). Further, incubation at 50 °C for 24 h (curve f) produces a further shift of the two peaks toward intermediate temperatures, 29 and 36 °C. The complete mixture sonicated gives a main peak at 34.5 °C. Incubation at temperatures lower than 50 °C, including at the $T_c$ of each of the components, generally inhibited the shift of peaks shown in Fig. 7.

These results indicate that considerably more mixing occurs with sonicated vesicles than with non-sonicated vesicles of similar composition. However, the predominant effect on the thermotropic properties of the vesicles is a gradual shift of both peaks toward intermediate temperatures, without the appearance of a third peak. These findings can be interpreted as indicating either exchange diffusion of molecules between the two vesicle populations or preferential fusion of smaller vesicles with larger ones. In either case there would be a gradual enrichment of each type of vesicle with relatively fewer molecules from the other. This would result in a gradual decrease of the $T_c$ of one and increase of the $T_c$ of the other, and the production of a one-to-one mixture only after prolonged incubation at temperatures above the $T_c$ of each original membrane. These results thus raise serious questions concerning the validity of earlier claims that myristic acid can induce fusion of phosphatidylcholine vesicles [10].

The results of these earlier studies [9] can be explained equally well on the basis of molecular diffusion with resulting formation of larger vesicles at the expense of the smaller ones. Furthermore, this enhanced exchange of molecules between membranes would appear to be related to sonication and the formation of "strained" vesicles and thus does not reflect an inherent characteristic of membranes of this composition.

*Effect of lysolecithin on the interaction of phospholipid vesicles.*

Lysolecithin has been shown by several investigators to be able to induce fusion of cellular membranes [26–28] and it was of interest therefore to determine what effect lysolecithin might have on the interaction of vesicles prepared from pure phospholipids. Fig. 8A shows the effects of lysolecithin, when added to preformed non-sonicated multilamellar vesicles of dimyristoyl phosphatidylcholine (curve c) and dipalmitoyl phosphatidylcholine (curve d) at a molar ratio of 1 to 4. Comparison of these curves with those obtained with the pure phospholipids (curves a and b) indicates that lysolecithin increases the midpoint $T_c$ by 1.5 °C for dimyristoyl phosphatidylcholine and by 2.2 °C for dipalmitoyl phosphatidylcholine and also produces considerable broadening of the transition region. This increase in $T_c$ was an unexpected result which does not support the impression derived from the literature (without supporting data) that lysolecithin is able to increase the "fluidity" of membrane lipids [29, 30].

As stated earlier, incubation of pure dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine non-sonicated vesicles does not produce appreciable molecular mixing during 1 h at 45 °C (Fig. 8A, curve e). If, however, the same vesicles are incubated under the same conditions but with lysolecithin added to the incubation mixture, considerable molecular mixing is obtained (Fig. 8B; curves a and b). Comparison of curve a with those of the pure lipids shows that lysolecithin induces the appearance of two new shoulder peaks at intermediate temperatures, with very little

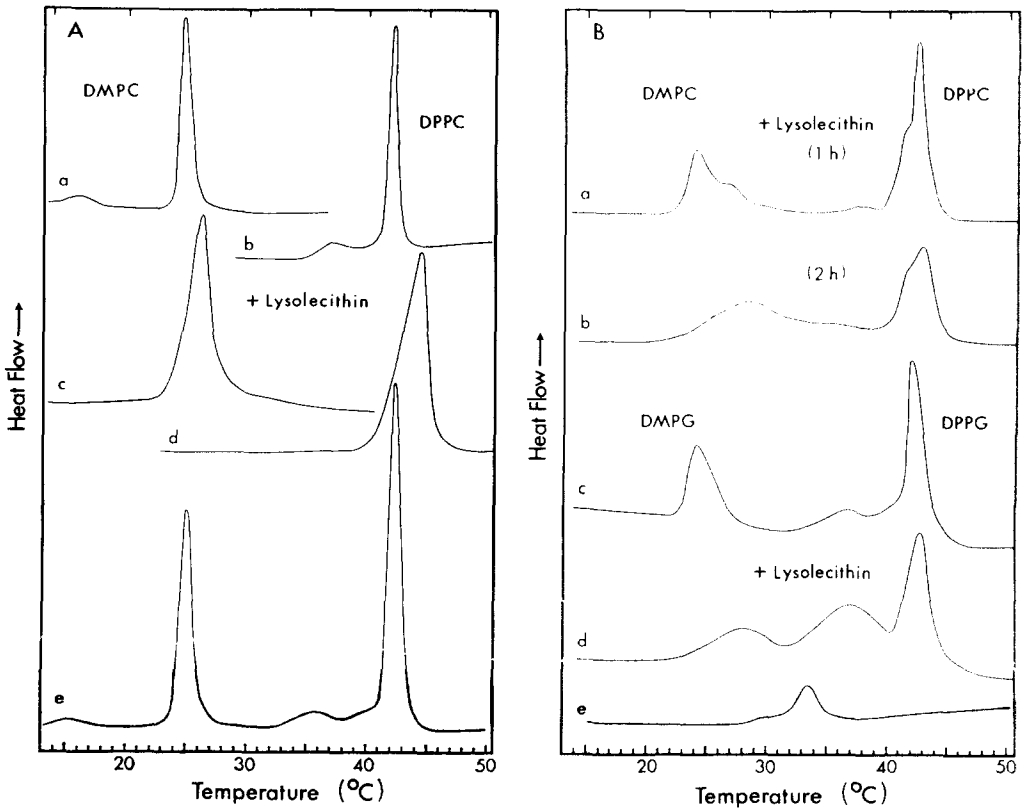


Fig. 8. Effect of lysolecithin on the thermotropic properties and molecular mixing in phospholipid vesicles. A: Individual phosphatidylcholine components were suspended separately in 100 mM NaCl buffer pH 7.4 at 45 °C and 10 μmol/ml. Lysolecithin was dissolved in the same buffer and was added in a ratio of 1.0 μmol to 4.0 μmol lipid in 0.5 ml total volume. Samples were incubated for 1 h at 45 °C: (a) dimyristoyl phosphatidylcholine alone; (b) dipalmitoyl phosphatidylcholine alone, (c) dimyristoyl phosphatidylcholine plus lysolecithin; (d) dipalmitoyl phosphatidylcholine plus lysolecithin; and (e) dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine formed separately and incubated for 1 h at 45 °C. B: Individual phospholipid vesicles prepared as in A and incubated as follows: (a) dimyristoyl phosphatidylcholine incubated with dipalmitoyl phosphatidylcholine in the presence of lysolecithin as in A (c and d) at a molar ratio of 1.0 to 4.0 total lipid, for 1 h at 45 °C; (b) same as in (a) except 2 h at 45 °C; (c) dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol formed separately and incubated together in equimolar amounts as in Fig. 4(a) for 1 h at 45 °C; (d) as in (c) above except for the addition of 2 μmol lysolecithin to 4 μmol of dimyristoyl phosphatidylglycerol and 4 μmol dipalmitoyl phosphatidylglycerol in 5 ml volume of 100 mM NaCl buffer; (e) as in (d) except for the addition of 5 μmol lysolecithin to 4 μmol dimyristoyl phosphatidylglycerol and 4 μmol of dipalmitoyl phosphatidylglycerol.

material melting in the temperature region of the complete mixture (34 °C). This indicates lysolecithin enhances mixing of vesicle membrane components via diffusion rather than fusion. Curve e (Figure 8B) shows that after 2 h incubation of the same mixture, there is considerable material melting within a broad temperature region between the two main peaks. This indicates considerable mixing that could be due to fusion as well as exchange diffusion. A similar experiment was undertaken with

populations of dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol vesicles. As shown in Fig. 8B (curves c, d and e) addition of 2 μmol of lysolecithin to 4 μmol of dimyristoyl phosphatidylglycerol and 4 μmol of dipalmitoyl phosphatidylglycerol (curve d) produced a pronounced middle peak, with a considerable shift of both initial peaks toward higher temperatures. The addition of 5 μmol of lysolecithin to the same mixture produced a complete mixing of the components as indicated by the presence of a single peak at 33.5 °C. Under these conditions, however, most of the lipid is solubilized and only a small fraction (approximately 20 %) can be sedimented by centrifugation at $100000 \times g$ for 30 min. The same solubilization effect was also noted in experiments using phosphatidylcholine vesicles exposed to similar lysolecithin concentrations. It is concluded that lysolecithin can produce some fusion at least with the phosphatidylglycerol vesicles but the initial effect on phosphatidylcholine membranes is an enhancement of the rate of molecular mixing probably via diffusion rather than fusion. It is also concluded that at relatively low concentrations of lysolecithin, before solubilization becomes pronounced, its effect on lipid bilayers is not "fluidization" but rather "stabilization" as evidenced by an increase in the transition temperature.

*Effect of a hydrophobic membrane protein on the interaction of phospholipid vesicles*

It was recently reported from this laboratory that incorporation of the major apoprotein from myelin proteolipid had very little effect on the $T_c$ of phospholipid membranes [24]. The apoprotein was incorporated by mixing the dry powder in chloroform/methanol/water with phospholipid in 15 % protein per weight basis, evaporated to dryness, and suspended in 10 mM NaCl buffer at pH 6.5 [24]. The protein was incorporated into either dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine vesicles and the lipoprotein membrane preparations incubated either separately or together at 45 °C for different periods of time. Curves a and b in Fig. 9 were obtained from these membranes incubated separately for 1 h. As reported elsewhere [24], the peaks are relatively broader, compared to the pure lipids, but the $T_c$ is the same as that of the pure lipid. Incubation of vesicles together for 30 min (curve c) and 90 min (curve d) revealed considerable molecular mixing with formation of a relatively large amount of material melting at intermediate temperatures. The main characteristic of this system is a gradual shift of the lower (dimyristoyl phosphatidylcholine) peak toward higher temperatures, with a simultaneous diminution of the dipalmitoyl phosphatidylcholine peak.

Although the above results clearly show substantial enhancement of the rate of molecular mixing between membranes in the presence of the apoprotein, it is not clear whether this is the result of fusion or exchange diffusion. The time sequence of the observed changes indicate, however, that small but increasing amounts of dipalmitoyl phosphatidylcholine molecules interact and mix with the bulk of the dimyristoyl phosphatidylcholine membranes, which slowly acquire a higher $T_c$. This is the opposite to that observed in Fig. 4, in which the more soluble component (dimyristoyl phosphatidylcholine) was diluting dipalmitoyl phosphatidylcholine membranes. It is therefore difficult to explain the observations in terms of diffusion of free molecules. The results can, however, be explained in terms of fusion between the two membranes if the assumption is made that the propensity to fuse is different for the various membranes present. The most probable sequence in terms of de-

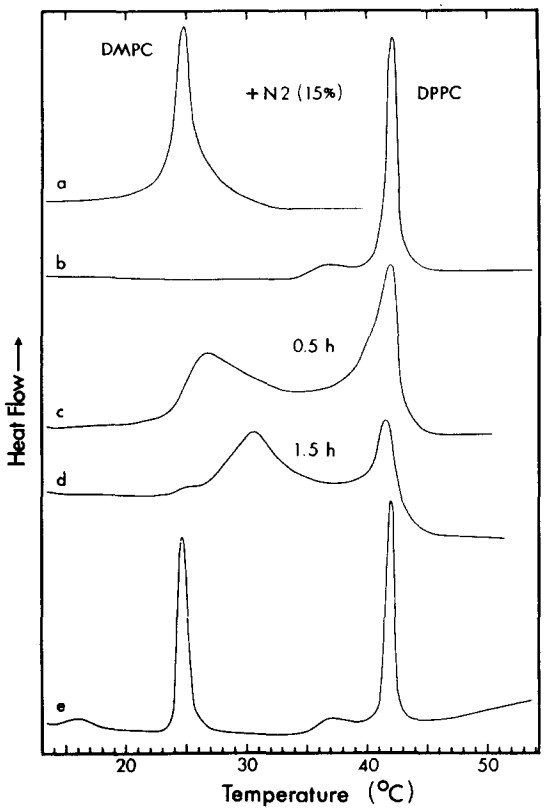


Fig. 9. Effect of a hydrophobic membrane protein on molecular mixing between phosphatidylcholine vesicles. Phospholipid was mixed with the major proteolipid apoprotein from myelin (N-2) in chloroform/methanol/water, evaporated together and suspended in 10 mM NaCl buffer pH 6.5 by shaking with the aid of glass beads at 45 °C. 2.7 mg of the apoprotein were mixed with 20 mol of each phosphatidylcholine and suspended in 4 ml buffer. The suspension was layered over a cushion of 60 % sucrose solution and centrifuged at 30 000 rev./min for 1 h at 20 °C. The material collected at the interface between sucrose and buffer was pipetted off, diluted to 4 ml of buffer and then incubated alone or after mixing at 45 °C as follows: (a) dimyristoyl phosphatidylcholine with N-2 alone 1 h; (b) dipalmitoyl phosphatidylcholine with N-2 alone, 1 h; (c) dimyristoyl phosphatidylcholine with N-2 and incubated together with dipalmitoyl phosphatidylcholine with N-2 for 0.5 h; (d) same mixture as in (c), incubated for 1.5 h; (e) pure dimyristoyl phosphatidylcholine vesicles incubated with pure dipalmitoyl phosphatidylcholine vesicles for 1.6 h at 45 °C.

creasing probability of fusion would be dimyristoyl phosphatidylcholine → dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine → dipalmitoyl phosphatidylcholine, a sequence which follows the increasing $T_c$ of these three membranes. It follows from this sequence that a mixed membrane (product of initial fusion between dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine) would have a higher probability to fuse with pure dimyristoyl phosphatidylcholine than with pure dipalmitoyl phosphatidylcholine. The result would then be continuous dilution of the dimyristoyl phosphatidylcholine membranes by dipalmitoyl phosphatidylcholine molecules observed in Fig. 9.

*The effect on dimethylsulfoxide on the interaction of phospholipid vesicles*

Recent observations on the effect of dimethylsulfoxide in causing topographic redistribution of intramembranous particles [31] and in promoting fusion of erythrocytes [32] have stimulated interest in the effect of this agent on phospholipid membranes. The effect of dimethylsulfoxide on the phase transition of pure phospholipid membranes has been studied in this laboratory in relation to the ability of this agent to induce erythroid cell differentiation [33]. This work established that dimethylsulfoxide produces an increase in the $T_c$ of phospholipid membranes indicating stabilization, perhaps through increased hydrogen bonding at the lipid-water interface. Dimethylsulfoxide produced only a 2–3 °C increase in the $T_c$ for dimyristoyl phosphatidylcholine membranes but a 20–30 °C increase in $T_c$ of acidic dimyristoyl phosphatidylglycerol membranes.

The effect of dimethylsulfoxide on phosphatidylcholine and phosphatidyl-

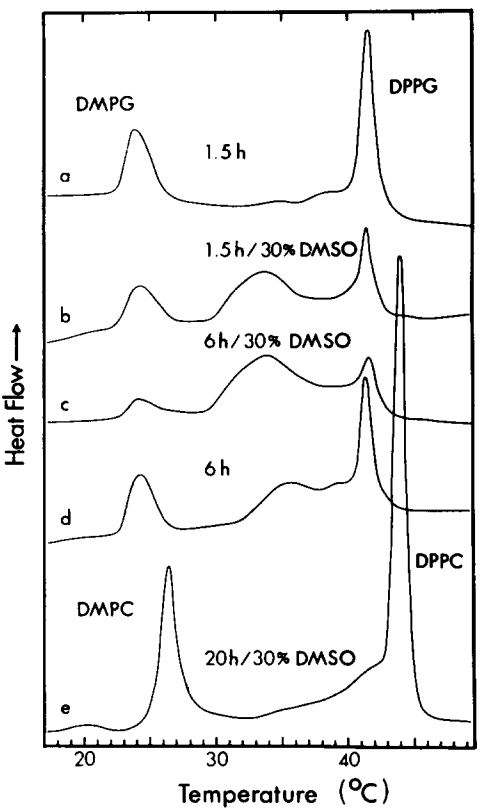


Fig. 10. Effect of dimethylsulfoxide on molecular mixing between phospholipid vesicles: (a) dimyristoyl phosphatidylglycerol (DMPG) vesicles and dipalmitoyl phosphatidylglycerol (DPPG) vesicles formed separately in 100 mM NaCl buffer, pH 7.4 as in Fig. 4 and incubated together at 45 °C for 1 h; (b) as in (a) except for the presence of 30 % dimethylsulfoxide (DMSO) (v/v); (c) as in (b) except for 6 h incubation with dimethylsulfoxide (d) as in (a), but the two vesicle populations were incubated together for 6 h at 45 °C; (e) dimyristoyl phosphatidylcholine (DMPC) vesicles and dipalmitoyl phosphatidylcholine (DPPC) vesicles formed separately (10 $\mu$mol/ml in 100 mM NaCl buffer, pH 7.4) and incubated together in the presence of 30 % dimethylsulfoxide for 20 h at 45 °C.

glycerol membranes is shown in Fig. 10. Multilamellar vesicles were formed in 100 mM (v/v) NaCl pH 7.4 and then incubated in 5–30 % dimethylsulfoxide (v/v) at 45 °C for different periods of time. The results obtained with dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles (curve e) indicated that incubation in 30 % dimethylsulfoxide for 20 h at 45 °C had no appreciable effect on the thermotropic transition of the individual components (curve e). As discussed earlier, this is direct evidence for lack of significant molecular mixing between the membranes and indicates that dimethylsulfoxide does not enhance fusion of these particular vesicles. However, the results obtained with the phosphatidylglycerol membranes revealed considerable mixing (Fig. 10). The membranes were centrifuged in the presence of dimethylsulfoxide at the end of incubation time period and resuspended in the initial buffer, before final centrifugation for calorimetric analysis. Curve a was obtained with dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol vesicles incubated together for 1.5 h without dimethylsulfoxide. Curves b and c show the effect of incubation with 30 % dimethylsulfoxide (v/v) at 45 °C for 1.5 and 6 h, respectively. The results indicate that dimethylsulfoxide induces a considerable increase in the rate of mixing as compared to the results shown in curves a and d without dimethylsulfoxide with the same membranes. Furthermore, the time sequence for the observed changes indicates that fusion rather than molecular diffusion is responsible for the mixing since the third intermediate peak at 33.5 °C increases in size while the two original peaks decrease in size without any gradual change in $T_c$ or shifting toward intermediate temperatures.

Thus, dimethylsulfoxide which generally appears to stabilize phospholipid bilayers [33] can enhance the rate of fusion of acidic phospholipid membranes in the absence of proteins. Furthermore, this effect does not appear to be related to fluidization of the bilayer as suggested by Lucy and colleagues [29, 32] but results probably from the ability of dimethylsulfoxide to induce a phase transition on acidic phospholipid membranes [33].

*General discussion and conclusions*

In this study we have used differential scanning calorimetry to monitor molecular mixing between two populations of phospholipid vesicles. The phospholipids chosen for this study each have distinct phase transition points but when mixed in organic solvent and dispersed in water together they form mixed membranes with a well defined transition point at a temperature intermediate to those of the individual components. Any molecular mixing that might occur during the incubation of the two vesicle populations can thus easily be identified. Calorimetric analysis of the mixing process has the important advantage that the kinetics of mixing can be used to differentiate between the two different mechanisms that produce mixing, namely, fusion of complete vesicles or exchange of individual phospholipid molecules between the membranes by diffusion.

The results presented here indicate that no appreciable fusion can be detected between non-sonicated phospholipid vesicles prepared from pure phospholipids in aqueous NaCl solutions. This statement applies to both neutral phosphatidylcholine and negatively charged phosphatidylglycerol vesicles, though molecular mixing via diffusion was observed with the latter. Mixing via exchange diffusion was enhanced when the vesicles contained surface charges of opposite signs, and was also enhanced

by the addition of lysolecithin. Addition of myristic acid had no appreciable effect on the rate of molecular exchange in non-sonicated phosphatidylcholine vesicles.

The question whether fusion occurs between sonicated phospholipid vesicles is more difficult to answer unequivocally. However, our data indicate that the increase in vesicle size observed here and in other studies [9–11, 14, 15] can be explained equally well by diffusion of free molecules between vesicles or by vesicle fusion. Similar evidence for molecular diffusion rather than vesicle fusion was obtained recently in a study with sonicated phosphatidylcholine vesicles by Martin and MacDonald [15]. It would appear that the increase in size in a given population of vesicles, with subsequent changes in the NMR spectrum or calorimetric signal, could be achieved by preferential diffusion of molecules from the smaller, more unstable vesicles, to the larger ones which accumulate in numbers and increase in size.

In view of the apparent resistance of pure neutral phosphatidylcholine vesicles to fusion reported here and elsewhere [8, 10, 15], it is of interest to note that such vesicles are also unable to fuse with the plasma membrane of living cells [34] or to produce fusion between cells [35]. In contrast, charged vesicles composed of lipids that are fluid at experimental temperatures are not only able to fuse with each other but can also fuse with the plasma membrane of living cells [34]. This striking difference in the fusion susceptibility of neutral and charged membranes suggests that some form of charge-charge interactions may be required in order to bring lipid bilayers into sufficiently close apposition for fusion to subsequently occur. Some support for this possibility is provided by recent calculations based on X-ray diffraction data [36] which indicate that the energy required to bring two neutral lecithin bilayers into close apposition (15 Å separation) is approximately 10 kT. Clearly, this energy barrier could not be overcome by the simple Brownian movement (1.5 k$T$).

The only case in which we have obtained evidence for fusion between phospholipid membranes in the absence of $Ca^{2+}$ resulted from incubation of vesicles with a hydrophobic protein from myelin, lysolecithin, or dimethylsulfoxide. The hydrophobic protein (N-2) is the major apoprotein from myelin proteolipid [25] and was shown recently to incorporate into phospholipid bilayers without large perturbations in the packing of the acyl chains [24]. Its effect in enhancing fusion is unique since as indicated above, phosphatidylcholine vesicles appear to be highly resistant to fusion under a variety of circumstances. The possible physiological implications of promotion of fusion of phosphatidylcholine membranes by a specific protein or peptide are presently unclear [14, 38]. Further studies are clearly required to understand the mechanism underlying this phenomenon [14, 38].

The effect of dimethylsulfoxide in promoting fusion between phosphatidylglycerol but not between phosphatidylcholine membranes, seems to be related to its ability to induce a phase transition with a $T_c$ at much higher temperatures [33]. The possible role of phase transitions in enhancing fusion between phospholipid vesicles has been discussed by us elsewhere [8, 12, 37]. Although further studies with dimethylsulfoxide are needed before its mechanism of action is understood, its effect is reminiscent of the effect of $Ca^{2+}$ in promoting vesicle fusion which is discussed extensively in the next two papers of this series. Thus, $Ca^{2+}$ induces the occurence of a phase transition and also induces fusion of acidic phospholipid membranes but not of neutral lecithin membranes [12, 37].

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