Findsen, E. W., Simons, P., & Ondrias, M. R. (1986) *Biochemistry 25*, 7912.
Friedman, J. M. (1985) *Science (Washington, D.C.) 228*, 1273-1280.
Friedman, J. M., & Lyons, K. B. (1980) *Nature (London) 284*, 570-579.
Friedman, J. M., Rousseau, D. L., Ondrias, M. R., & Stepnoski, R. A. (1982a) *Science (Washington, D.C.) 218*, 1244-1246.
Friedman, J. M., Ondrias, M. R., & Rousseau, D. L. (1982b) *Annu. Rev. Phys. Chem. 33*, 471.
Friedman, J. M., Scott, T. W., Stepnoski, R. A., Ikeda-Saito, M., & Yonetani, J. (1983) *J. Biol. Chem. 258*, 10564-10573.
Friedman, J. M., Scott, T. W., Fisanick, G. J., Simon, S. R., Findsen, E. W., Ondrias, M. R., & MacDonald, V. R. (1985) *Science (Washington, D.C.) 229*, 187.
Gelin, B. R., & Karplus, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 801-805.
Gelin, B. R., Lee, A. W.-M., & Karplus, M. (1983) *J. Mol. Biol. 171*, 489-559.
Gibson, Q. H., Olson, J. S., McKinnie, R. E., & Rohlfs, G. (1986) *J. Biol. Chem. 261*, 10228-10229.
Gurd, F. R. N., & Rothgeb, T. J. (1979) *Adv. Protein Chem. 33*, 74.
Hartman, H., Park, F., Steigeman, W., Petsko, G. A., Ponzi, D. R., & Frauenfelder, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4967.
Hasinoff, B. B. (1981) *J. Phys. Chem. 85*, 528-531.
Hasinoff, B. B., & Chishti, S. B. (1982) *Biochemistry 21*, 4275-4278.
Hasinoff, B. B., & Chishti, S. B. (1983) *Biochemistry 22*, 58-61.
Henry, E. R., Levitt, M., & Eaton, W. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2034-2038.
Hofrichter, J., Sommer, J. H., Henry, E. R., & Eaton, W. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2235.
Karplus, M., & McCammon, A. (1983) *Annu. Rev. Biochem. 53*, 263-300.
Lesk, A. M., & Chothia, C. (1984) *J. Mol. Biol. 174*, 175-191.
Lyons, K. B., Friedman, J. M., & Fleury, P. A. (1978) *Nature (London) 275*, 565-566.
Martin, J. L., Nigus, A., Poyant, C., Lecarpentier, Y., Astier, R., & Antonetti, A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 173.
Morris, R. J., & Gibson, Q. H. (1984) *J. Biol. Chem 259*, 367-374.
Ondrias, M. R., Friedman, J. M., & Rousseau, D. L. (1983) *Science (Washington, D.C.) 220*, 615-619.
Perutz, M. F. (1970) *Nature (London) 228*, 726-734.
Rousseau, D. L., & Ondrias, M. R. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 357-380.
Rousseau, D. L., & Friedman, J. M. (1988) in *Biological Application of Raman Scattering* (Spiro, T. G., Ed.) Vol. III, Wiley, New York.
Scott, T. W., & Friedman, J. M. (1984) *J. Am. Chem. Soc. 106*, 5677-5687.
Spiro, T. G. (1983) in *Iron Porphyrins* (Lever, A. B. P., & Gray, H. B., Eds.) Vol. II, pp 89-139, Addison-Wesley, Reading, MA.
Stein, P., Terner, J., & Spiro, T. G. (1982) *J. Phys. Chem. 86*, 168-172.
Terner, J., Stong, J. D., Spiro, T. G., Nagumo, M., Nicol, M. S., & El-Sayed, M. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1313-1317.

Spontaneous Vesiculation of Large Multilamellar Vesicles Composed of Saturated Phosphatidylcholine and Phosphatidylglycerol Mixtures†

T. D. Madden,*,‡ C. P. S. Tilcock, K. Wong, and P. R. Cullis‡
Department of Biochemistry, The University of British Columbia, Vancouver, British Columbia V6T 1W5, Canada
Received May 3, 1988; Revised Manuscript Received July 11, 1988

ABSTRACT: The influence of temperature and ionic strength on the vesiculation properties of large multilamellar vesicles containing various proportions of dimyristoylphosphatidylglycerol has been investigated. It is shown that at low ionic strengths preformed large multilamellar vesicles composed of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol (7:3) on incubation at the gel to liquid-crystalline transition temperature (T_c ~23 °C) spontaneously vesiculate to form predominantly unilamellar systems with a mean diameter of 120 nm. Such vesiculation is not observed for incubations at temperatures appreciably above or below T_c, and is also inhibited by higher ionic strengths. Stable large multilamellar vesicles are formed, however, in systems containing the dioleoyl species of phosphatidylcholine or phosphatidylglycerol and also for dimyristoylphosphatidylcholine/dimyristoylphosphatidylserine mixtures. The vesiculation properties of dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol mixtures, therefore, appear to reflect an instability in the region of the T_c driven by surface potential effects which are specific for the glycerol headgroup.

Upon hydration, most naturally occurring phospholipids adopt either the bilayer organization or the hexagonal H_{II} phase (Cullis & de Kruijff, 1979; Cullis et al., 1985). In both instances, the macromolecular structures formed are large (several micrometers) and stable such that even transitions between these polymorphic phases do not generate small vesicles. One exception is the case of cardiolipin which in the presence of calcium adopts the hexagonal H_{II} phase. If this mixture is dialyzed against EDTA, small vesicles are generated

† This research was supported by the Medical Research Council of Canada and The Liposome Co., Princeton, NJ.
‡ Present address: The Canadian Liposome Co., Ltd., Suite 308, 267 West Esplanade, North Vancouver, British Columbia V7M 1A5, Canada.

0006-2960/88/0427-8724$01.50/0

(Vail & Stollery, 1979). However, this is presumably due to the removal of calcium from cardiolipin at the exterior of the cylindrical H_{II} arrays and the consequent "blebbing-off" of bilayer vesicles. Although large multilamellar vesicles (MLVs)[1] formed by the hydration of dry lipid are under osmotic stress due to nonequilibrium solute distribution (Gruner et al., 1985; Mayer et al., 1986), they are nevertheless stable structures. The formation of LUVs or SUVs from MLVs usually requires aggressive disruption, for example, by sonication (Huang, 1969) or extrusion through polycarbonate filters (Hope et al., 1985). While the formation of LUVs from mixtures of phosphatidylcholine with either charged single-chain detergents (Hauser et al., 1986) or short-chain phospholipids (Gabriel & Roberts, 1984) has been described, the only reported instance of MLVs composed solely of bilayer-forming phospholipids spontaneously vesiculating concerns mixtures of acidic phospholipids and phosphatidylcholine transiently exposed to an alkaline pH (Hauser & Gains, 1982; Gains & Hauser, 1983; Li & Haines, 1986). We show here that a similar process can occur at neutral pH for mixtures of saturated phosphatidylcholine and phosphatidylglycerol. Surprisingly, vesiculation is rapid only at temperatures around the gel to liquid-crystalline phase transition.

Materials and Methods

Dimyristoylphosphatidylcholine, dimyristoylphosphatidylglycerol, dimyristoylphosphatidylserine, dioleoylphosphatidylcholine, and dioleoylphosphatidylglycerol were purchased from Avanti Polar Lipids (Birmingham, AL). Lipid purity was determined by thin-layer chromatography using a solvent system of $CHCl_3/MeOH/NH_4OH/H_2O$ (900:540:5.7:5.3 v/v), and the lipids were detected by charring. All the lipid species showed a single spot on heavily loaded plates.

Formation of Lipid Vesicles. Lipid mixtures were colyophilized from benzene/methanol (70:30 v/v), and the dry lipid was stored at −20 °C. Unless otherwise stated, the lipid was hydrated on ice in 2 mM HEPES, pH 7.6, at a concentration of 10 mM phospholipid. The mixture was then transferred to a thermostated water bath and gently stirred. For the Mn^{2+} permeability experiment, large unilamellar vesicles were prepared by using the Extruder (Lipex Biomembranes, Inc., Vancouver, B.C.) as detailed previously (Hope et al., 1985; Mayer et al., 1986).

Quasi-Elastic Light Scattering. Vesicle size distributions were determined by using a Nicomp particle sizer (Nicomp Instruments, Goleta, CA) operating at 632.8 nm and 5 mW. This apparatus is optimized to determine particle sizes of less than about 1 μm, and where mean diameters of greater than 1 μm are given, the value should be considered to be an approximation. Aliquots of the lipid suspension were diluted 1:10 with 150 mM NaCl/10 mM HEPES, pH 7.6, which had previously been filtered through a 0.2-μm polycarbonate filter (Nuclepore Corp.). Measurements were made at 22 °C.

Nuclear Magnetic Resonance Spectroscopy. Samples for ^{31}P NMR contained 10% D_2O and lipid concentrations of 10 mM. Generally, spectra were obtained by employing a Bruker WP-200 Fourier-transform spectrometer, but the sample DMPC/DMPS (7:3) was examined with a Varian XL300 instrument. Accumulated free induction decays were obtained for up to 5000 transients using an interpulse time of 1 s, a 16-μs 90° pulse, and a sweep width of up to 50 kHz. Spectra were generally recorded at 30 °C in the presence of broad-band proton decoupling.

Freeze–Fracture Electron Microscopy. Samples contained 25% glycerol as a cryoprotectant and were quenched from 30 °C in liquid Freon and then fractured and replicated at −110 °C and $<10^{-6}$ torr employing a Balzers BAF 400 freeze–fracture unit. Replicas were cleaned in chromic acid and visualized in a Jeol JEM-1200 EX microscope.

Differential Scanning Calorimetry. Samples were sealed in 75-μL stainless-steel pans and inserted into a Perkin-Elmer DSC 2C calorimeter at 0 °C. Heating rates of 2 °C min^{-1} were used, and thermograms were recorded at least twice. In the case of DMPC/DOPG, DOPC/DMPG, and DOPC/DMPC/DOPG/DMPG mixtures, the lipids were hydrated in 2 mM HEPES, pH 7.6, containing 28 wt % ethylene glycol and then scanned from −10 to 35 °C. These samples were then cooled at 5 °C min^{-1} back down to −10 °C and rescanned. In addition, the referenced pan contained an approximately equal weight of Sephadex G-50 swollen in 28 wt % ethylene glycol.

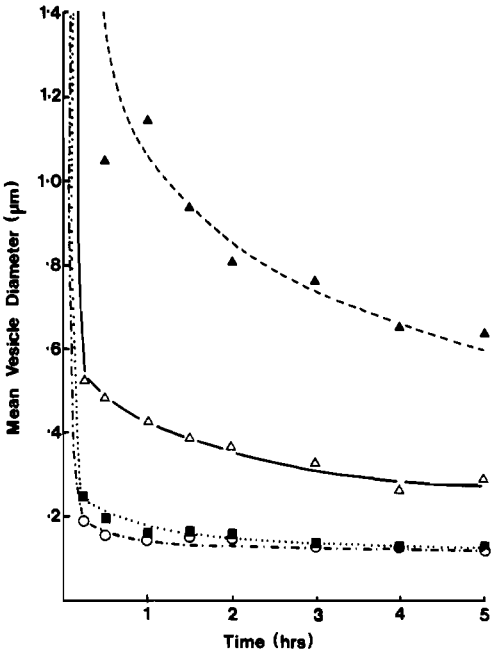

FIGURE 1: Vesiculation of DMPC/DMPG (7:3) MLVs as a function of ionic strength. DMPC/DMPG (10 mM) was hydrated at 4 °C in the media shown below and incubated at 24 °C. Samples were in H_2O (○), 2 mM HEPES, pH 7.6 (■), 10 mM NaCl/2 mM HEPES, pH 7.6 (△), or 25 mM NaCl/2 mM HEPES, pH 7.6 (▲).

Results

When DMPC/DMPG (7:3) is hydrated at 4 °C, it forms MLVs irrespective of the ionic strength of the hydrating medium. If this mixture is then incubated at 24 °C, however, while the MLVs dispersed in 150 mM NaCl/10 mM HEPES, pH 7.6, are stable, those formed in H_2O spontaneously vesiculate. This phenomenon is illustrated in Figure 1 where the mean vesicle diameter as a function of incubation time is shown for lipid dispersed at various ionic strengths. In the presence of H_2O alone or 2 mM HEPES, pH 7.4, rapid vesiculation occurs with mean vesicle diameters of approximately 200 nm observed after 15 min at 24 °C. With further incubation, vesicle size continues to decline although much less rapidly with a mean diameter of about 120 nm observed after 24 h.

[1] Abbreviations: MLVs, large multilamellar vesicles; LUVs, large unilamellar vesicles; SUVs, small unilamellar vesicles; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DMPS, dimyristoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid.

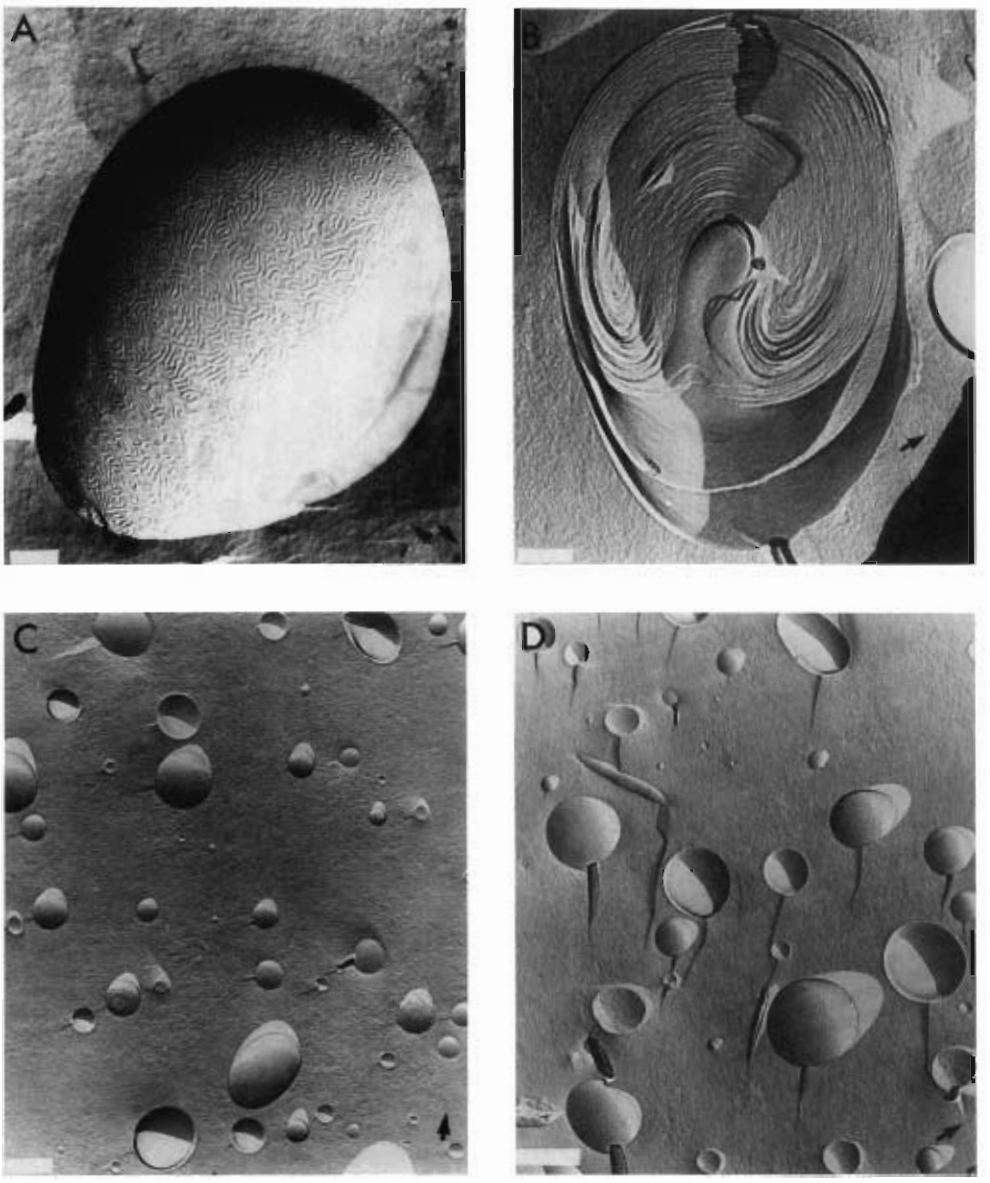

FIGURE 2: Electron micrographs of DMPC/DMPG (7:3) vesicles. Lipid (10 mM) was incubated at 24 °C for 24 h in 150 mM NaCl/10 mM HEPES, pH 7.6 (A and B), or in 2 mM HEPES, pH 7.6 (C and D). The bar represents 200 nm, and the direction of shadowing is indicated by the arrow in each micrograph.

If the ionic strength of the hydrating medium is increased, however, the rate of MLV breakdown is markedly reduced (Figure 1) with no vesiculation occurring in 150 mM NaCl. Following incubation at 24 °C for 24 h, samples were examined for lipid degradation products by thin-layer chromatography. In all cases, lysophosphilipid production amounted to less than 1%.

To confirm the light-scattering data, lipid samples were also examined by freeze–fracture electron microscopy and ^{31}P NMR. In Figure 2 are shown electron micrographs of DMPC/DMPG mixtures incubated for 24 h in 150 mM NaCl/10 mM HEPES, pH 7.6, or in 2 mM HEPES, pH 7.6. In high salt, large structures (≥ 1 μm) are seen, and occasional cross-fractures reveal the characteristic "onion rings" of multilamellar vesicles. In contrast, the lipid mixtures incubated in low-salt medium show numerous vesicles ranging in size between 35 and 400 nm with a mean of about 120 nm. In this sample, only rarely do cross-fractures show the presence of internal lamellae.

The ^{31}P NMR spectrum of DMPC/DMPG hydrated on ice in 2 mM HEPES, pH 7.6, shows a characteristic bilayer line shape with a low-field shoulder and two high-field peaks arising from the phosphatidylcholine and phosphatidylglycerol headgroups (Figure 3A). The width of the spectrum and the absence of an isotropic signal indicate that all of the vesicles must be at least 400 nm in diameter (Burnell et al., 1980). Following incubation of the mixture at 24 °C for 1 h, however, the width of the spectrum is substantially reduced with the peak now centered at the resonance position characteristic of isotropic lipid motion (Figure 3B). Such a signal would be expected for vesicles smaller than 400 nm and arises due to the rapid lateral mobility of the phospholipids and vesicle tumbling effects (Burnell et al., 1980). After 12-h incubation at 24 °C, a further reduction in the spectral width is observed, and separate resonances for the two lipid species can now be resolved (Figure 3C). The ^{31}P NMR data, therefore, are consistent with both light scattering and freeze–fracture electron microscopy and confirm that DMPC/DMPG (7:3) while initially hydrating to form MLVs spontaneously vesiculates upon incubation at 24 °C. The ^{31}P NMR spectrum of the same lipid mixture incubated for 12 h under the same conditions in 150 mM NaCl/10 mM HEPES, pH 7.6, shows no isotropic component (Figure 3D), confirming that vesiculation does not occur in high ionic strength media.

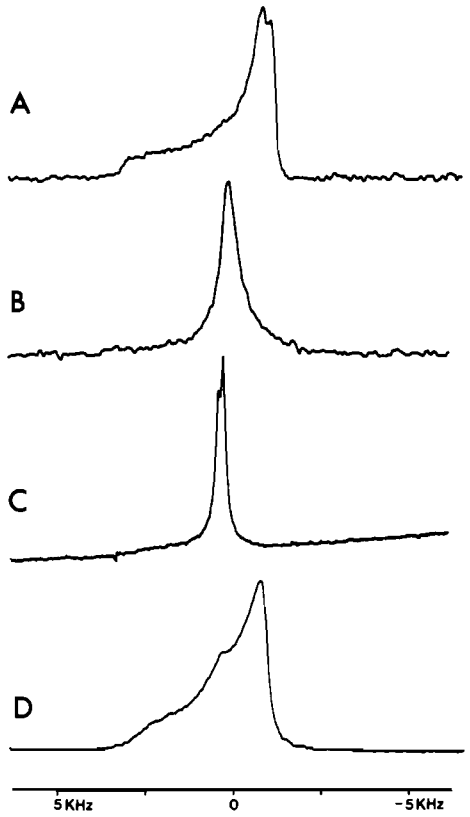

FIGURE 3: ^{31}P NMR of DMPC/DMPG (7:3). Lipid (10 mM) was hydrated in H_2O at 4 °C, and its spectrum was then recorded at 30 °C (A). The same lipid mixture was then incubated at 24 °C for 1 h (B) and 12 h (C). DMPC/DMPG (7:3) hydrated in 150 mM NaCl/10 mM HEPES, pH 7.6, and incubated at 24 °C for 12 h is shown in (D).

At 23 °C, DMPC/DMPG (7:3) undergoes a transition from the gel to liquid-crystalline state. To determine what influence the lipid phase may have on MLV stability, therefore, samples were incubated below (15 °C), above (32 °C), and within (24 °C) the transition temperature (T_c) region. For the sample maintained above T_c, the dry lipid was incubated for 1 h in a water-saturated atmosphere at 32 °C before the addition of excess 2 mM HEPES, pH 7.6, preincubated at the same temperature. This protocol was adopted because anhydrous DMPC is in the gel state at 32 °C (Chapman et al., 1967) and we wanted to ensure that the fully hydrated lipid did not pass through the phase transition. Remarkably, DMPC/DMPG MLVs hydrated well above or well below the gel to liquid-crystalline transition temperature show little reduction in mean vesicle diameter over a 6-h incubation while the same mixture held at 24 °C undergoes rapid vesiculation (Figure 4). In addition, MLVs incubated at 15 or 32 °C vesiculate to form LUVs when brought to 24 °C. These results suggest that vesiculation occurs much more rapidly when domains of both gel and liquid-crystalline lipid coexist. This would further imply that MLVs composed of unsaturated lipids such as dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) which have a transition temperature of approximately −20 °C (Ladbrooke & Chapman, 1969; Findlay & Barton, 1978) would be more stable at 24 °C than the myristoyl species. Such proves to be the case as shown by the ^{31}P NMR spectrum of DOPC/DOPG (7:3) incubated at 24 °C for 24 h in 2 mM HEPES, pH 7.6 (results not shown). While a small isotropic peak indicates that some vesiculation has occurred, the majority of the lipid still gives rise to a typical bilayer spectrum. The same lipid mixture incubated in high-salt media shows no isotropic component.

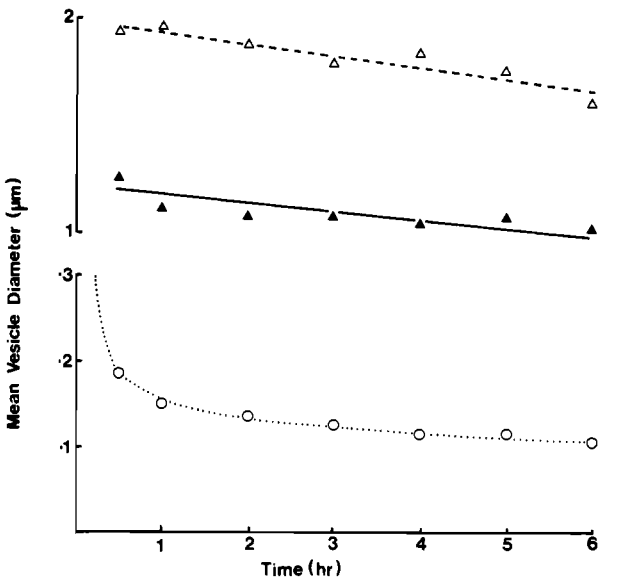

FIGURE 4: Influence of incubation temperature on the size of DMPC/DMPG (7:3) MLVs. Lipid (10 mM) was hydrated in 2 mM HEPES, pH 7.6, and incubated at 15 (▲), 24 (○), or 32 °C (△). For further details, see Results.

The question then arises as to whether phase domains which also cause segregation of the phosphatidylcholine and phosphatidylglycerol lead to a destabilization of MLV structure. To address this point, mixtures of DMPC/DOPG (7:3), DOPC/DMPG (7:3), and DOPC/DMPC/DOPG/DMPG (7:7:3:3) were prepared. Phosphatidylcholines and phosphatidylglycerols with the same fatty acyl composition show high miscibility (van Dijck et al., 1975; Findlay & Barton, 1978) as do mixtures of these lipids of differing acyl chain length or unsaturation when both are in the liquid-crystalline phase. Mixtures of dimyristoyl- and dioleoylphosphatidylcholine, however, show monotectic behavior where the higher melting component segregates into gel-state domains upon cooling below 24 °C. Consequently, on heating, two endotherms are recorded by differential scanning calorimetry corresponding to the two species of phosphatidylcholine (Ladbrooke & Chapman, 1969). Similar behavior is shown by mixtures of phosphatidylcholine and phosphatidylglycerol. A broad endotherm between −5 and 15 °C is seen for a DMPC/DOPG (7:3) mixture (Figure 5A) which is comparable to the higher melting component observed by Ladbrooke and Chapman (1969). In contrast, the DOPC/DMPG mixture shows only a very small endotherm in the same temperature region (Figure 5B). When both gel and liquid-crystalline domains contain both headgroups as in the case of the DOPC/DMPC/DOPG/DMPG mixture, a very broad endotherm extending from −5 to 25 °C is observed (Figure 5C). These three lipid mixtures were incubated at either 24 or 10 °C for 16 h in 2 mM HEPES, pH 7.6, or 150 mM NaCl/20 mM HEPES, pH 7.6, and then examined by ^{31}P NMR. As would be predicted from the data in Figure 1 for DMPC/DMPG, in high-salt medium all three mixtures show only a typical bilayer spectrum with no evidence of MLV breakdown (results not shown). When the samples are incubated at 10 °C, domains of both gel and liquid-crystalline lipid coexist; nevertheless, both at this temperature and at 24 °C, very little vesiculation is apparent as judged by the absence of any appreciable isotropic component in the ^{31}P NMR spectra of these mixtures (results not shown). This result would indicate that even when phase domains are present, with or without segregation of the negatively charged phospholipid, the presence of the dioleoyl species stabilizes MLV structure.

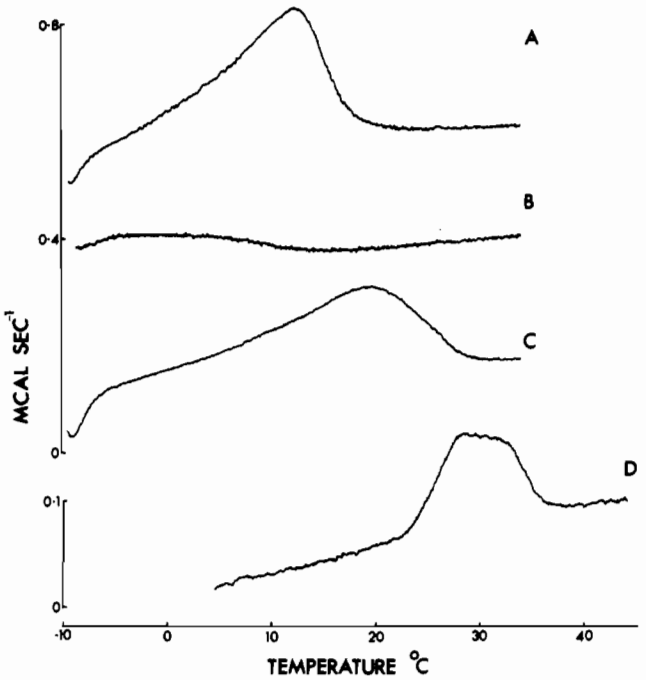

FIGURE 5: Differential scanning calorimetric analysis of phosphatidylcholine mixtures with phosphatidylgylcerol or phosphatidylserine. Heating thermograms are shown for DMPC/DOPG (7:3) (A), DOPC/DMPG (7:3) (B), DOPC/DMPC/DOPG/DMPG (7:7:3:3) (C), and DMPC/DMPS (7:3) (D). Analyses were performed as described under Materials and Methods.

To further characterize the influence of fatty acyl composition on the properties of phosphatidylcholine/phosphatidylglycerol mixtures, we examined the permeability of LUVs prepared from DMPC/DMPG (7:3), DOPC/DMPG (7:3), and DOPC/DMPC/DOPG/DMPG (7:7:3:3) to Mn^{2+}. This broadening agent effectively eliminates the ^{31}P NMR signal from phospholipid to which it has access. In the case of LUVs, this corresponds to the outer monolayer, and upon addition of Mn^{2+}, therefore, the ^{31}P NMR signal is reduced by about 50%. If vesicles are permeable to Mn^{2+}, however, a further reduction in signal is observed as the phospholipids of the inner monolayer are titrated. The ^{31}P NMR spectra of LUVs prepared by using the extrusion technique (see Materials and Methods) before and after addition of the broadening agent are presented in Figure 6. Clearly, even at 30 °C, well above the T_c of the lipid, vesicles composed of DMPC/DMPG are highly permeable, and virtually all of the phospholipids are accessible to Mn^{2+} (Figure 6A). Surprisingly, this high permeability is also observed for vesicles prepared in 150 mM NaCl (results not shown). It should be noted that these experiments were performed under conditions where MLVs composed of DMPC/DMPG are relatively stable (cf. Figure 4). Similar results to those shown for LUVs above were obtained when DMPC/DMPG MLVs were used. When Mn^{2+} is added to DOPC/DMPG or DOPC/DMPC/DOPG/DMPG LUVs, there is an intial reudction in signal intensity by 50–60% but little further change with time (Figure 6B,C), indicating that the vesicles are relatively impermeable to the cation. This would be consistent with the greater stability of MLVs composed of these mixtures.

The influence of salt concentration on the stability of DMPC/DMPG MLVs is consistent with charge repulsion between the acidic phosphatidylglycerol headgroups being a major factor causing vesiculation. To investigate this further, various ratios of DMPC and DMPG were hydrated in 10 mM NaCl/2 mM HEPES, pH 7.6, and incubated at 24 °C. A low concentration of NaCl was included in the medium to reduce the rate of vesiculation and allow comparisons to be made between the different mixtures. As expected, DMPC alone forms stable MLVs while increasing the ratio of DMPG to DMPC increases the rate of spontaneous vesiculation (Figure 7). To determine whether the nature of the negatively charged headgroup influences stability or whether charge repulsion alone is important, a mixture of DMPC and dimyristoylphosphatidylserine (DMPS) (molar ratio 7:3) was prepared. DMPS undergoes a gel to liquid-crystalline phase transition at about 38 °C while the 7:3 mixture shows a broad transition between 24.5 and 34 °C (Figure 5D). An equimolar mixture of these lipids shows two overlapping endotherms by DSC (result not shown), in agreement with a previous report (van Dijck et al., 1978) which has been interpreted as indicating gel phase immiscibility. Following hydration in 2 mM HEPES, pH 7.6, samples were incubated at 24, 33, or 40 °C for up to 12 h with no significant reduction in mean vesicle diameter (results not shown). The ^{31}P NMR spectra of DMPC/DMPS (7:3) after incubation at 30 °C for 12 h show no appreciable isotropic signal, confirming that all of the lipid is present in structures of diameter greater than 400 nm. When the permeability of DMPC/DMPS LUVs to Mn^{2+} is examined, however, we observed that, as for DMPC/DMPG, addition of the broadening reagent results in complete elimination of the ^{31}P NMR signal (Figure 6D), indicating ready access of the Mn^{2+} to the interior of the vesicles. Clearly,

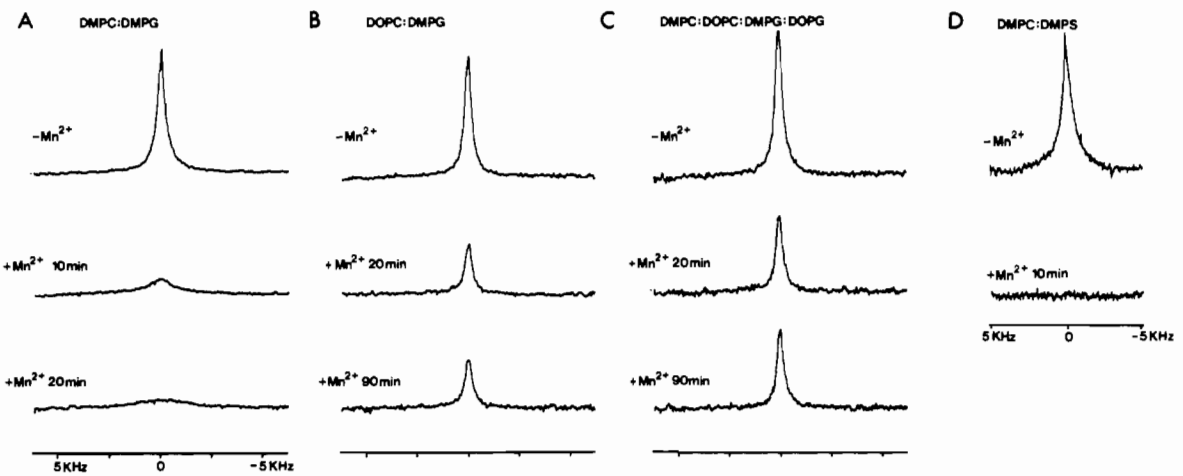

FIGURE 6: Influence of Mn^{2+} on the ^{31}P NMR spectrum of phosphatidylcholine with either phosphatidylglycerol or phosphatidylserine. LUVs of the mixtures shown were prepared at a lipid concentration of 10 mM as described under Materials and Methods. Spectra were obtained before and after the addition of 100 μM Mn^{2+} at the time intervals shown.

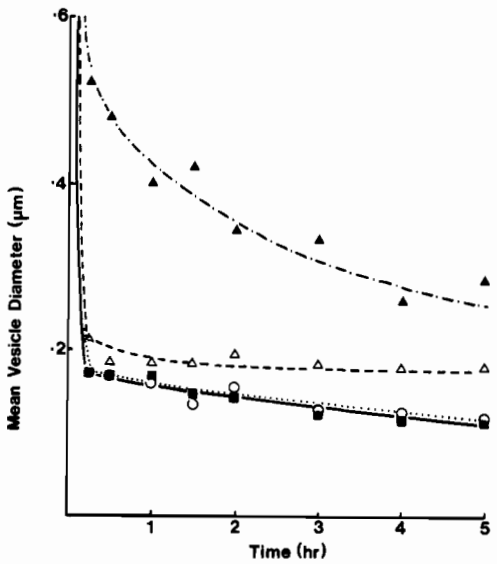

FIGURE 7: Influence of DMPC/DMPG molar ratio on the rate of MLV vesiculation. Lipid (10 mM) was hydrated at 4 °C in 10 mM NaCl/2 mM HEPES, pH 7.6, and then incubated at 24 °C. Samples consisted of DMPG (□), DMPC/DMPG (3:7) (○), DMPC/DMPG (1:1) (△), and DMPC/DMPG (7:3) (▲).

therefore, the nature of the acidic headgroup is important in determining MLV stability, and this point will be further addressed under Discussion.

Discussion

A striking feature of spontaneous vesiculation by DMPC/DMPG mixtures is that the process occurs rapidly only at the lipid phase transition temperature. It has previously been shown that the cation permeability of liposomes is also markedly enhanced in this temperature region (Haest et al., 1972; Papahadjopoulos et al., 1973) as is their susceptibility to hydrolysis by phospholipase A_2 (Op den Kamp et al., 1974, 1975). McConnell and co-workers have proposed that these and similar observations can be accounted for by increased lateral compressibility when domains of both gel and liquid-crystalline lipid coexist, allowing penetration of the bilayer with virtually no change in surface pressure (Linden et al., 1973). How such an increased compressibility would promote vesiculation, however, is unknown. Clearly, the mere presence of phase domains is insufficient to produce MLV breakdown whether or not phase separation also results in segregation of the phosphatidylcholine and phosphatidylglycerol. When mixtures of the dimyristoyl and dioleoyl species of these lipids are prepared, the higher melting component phase separates below its T_c, but irrespective of whether this constitutes the phosphatidylcholine or phosphatidylglycerol, little or no vesiculation is observed. Even when the gel and liquid-crystalline domains contain both phospholipid species, e.g., DMPC/DOPC/DMPG/DOPG (7:7:3:3), only very limited breakdown of MLV structure is apparent. Clearly, in these systems, the presence of dioleoylphospholipids stabilizes MLV structure.

The concept of charge repulsion between acidic phosphatidylglycerol headgroups providing the driving force for vesiculation is supported by two observations. First, DMPC alone forms stable MLVs while as the ratio of DMPG to DMPC is increased the rate of vesiculation also increases. Second, MLV stability is highly dependent upon ionic strength; as the salt concentration of the hydrating medium is increased, the rate of MLV breakdown is considerably reduced. The nature of the acidic headgroup, however, is also important. While both DMPC/DMPG and DMPC/DMPS vesicles are highly permeable to Mn^{2+}, only the phosphatidylglycerol-containing systems exhibit vesiculation. This difference may in part be due to the miscibility properties of these two lipids. Mixtures of DMPC/DMPG are ideally mixed both in the gel and in the liquid-crystalline state while calorimetric analysis of DMPC/DMPS mixtures shows separate endotherms for the two lipids in samples containing 66–80 mol % DMPS (van Dijck et al., 1978) and relatively broad single endotherms at lower phosphatidylserine ratios which was interpreted as indicating gel phase immiscibility.

The reasons why charge repulsion should tend to destabilize MLV structure resulting in vesiculation are not clear. One possibility is that smaller vesicles with consequently smaller radii of curvature are more stable. This could arise due to an asymmetric distribution of lipids with phosphatidylcholine preferentially located on the inner monolayer and phosphatidylglycerol in the outer monolayer. The larger headgroup spacing for the negatively charged phosphatidylglycerol would clearly correspond to a lower energy situation. While such an asymmetric distribution of these two lipids in SUVs has previously been reported (Michaelson et al., 1973; Lentz et al., 1980), other workers have presented contradictory findings (Barsukov et al., 1980; Nordlund et al., 1981). This explanation is underminded, however, by two experimental observations. First, the mean size of the vesicle population produced by vesiculation is greater than 100 nm, implying that only a small increase in curvature is experienced by the majority of vesicles. Prolonged incubation does not significantly reduce this mean size, but this may reflect mechanistic difficulties for vesiculation rather than the absence of a thermodynamic advantage for smaller systems. The second, perhaps more significant, observation is that DMPG alone rapidly vesiculates. Clearly, the generation of asymmetry cannot be the driving force in this system.

It has previously been shown that the interlamellar spacing in MLVs containing charged phospholipids is significantly greater than for zwitterionic species (Gulik-Krzywicki et al., 1969) due to interbilayer charge repulsion. This interaction is clearly a logical candidate for the driving force behind vesiculation as by generating unilamellar vesicles such repulsion is reduced.

Registry No. DMPC, 13699-48-4; DMPG, 61361-72-6; DOPC, 10015-85-7; DOPG, 62700-69-0.

References

Barsukov, L. I., Victorov, A. V., Vasilenko, I. A., Erstigneeva, R. P., & Bergelson, L. D. (1980) *Biochim. Biophys. Acta 598*, 153–168.

Burnell, E. E., Cullis, P. R., & deKruijff, B. (1980) *Biochim. Biophs. Acta 603*, 63–69.

Chapman, D., Williams, R. M., & Ladbrooke, B. D. (1967) *Chem. Phys. Lipids 1*, 445–475.

Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 339–420.

Cullis, P. R., de Kruijff, B., Verkleij, A. J., & Tilcock, C. P. S. (1985) in *Phospholipids and Cellular Regulation* (Kuo, J. F., Ed.) CRC, Boca Raton, FL.

Findlay, E. J., & Barton, P. G. (1987) *Biochemistry 17*, 2400–2405.

Gabriel, N. E., & Roberts, M. F. (1984) *Biochemistry 23*, 4011–4015.

Gains, N., & Hauser, H. (1983) *Biochim. Biophys. Acta 731*, 31–39.

Gruner, S. M., Lenk, R. R., Janoff, A. S., & Ostro, M. J. (1985) *Biochemistry 24*, 2833–2842.

Gulik-Krzywicki, T., Tardieu, A., & Luzzati, V. (1969) *Mol. Cryst. Liq. Cryst. 8*, 285–309.

Haest, C. W. M., de Gier, J. van Es, G. A., Verkleij, A. J., & van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 288*, 43-53.
Hauser, H., & Gains, N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1683-1687.
Hauser, H., Gains, N., Eible, H.-J., Muller, M., & Wehrli, E. (1986) *Biochemistry 25*, 2126-2134.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55-65.
Huang, C. H. (1969) *Biochemistry 8*, 344-352.
Ladbrooke, B. D., & Chapman, D. (1969) *Chem. Phys. Lipids 3*, 304-364.
Lentz, B. R., Alford, D. R., & Dombrose, F. A. (1980) *Biochemistry 19*, 2555-2559.
Li, W., & Haines, T. H. (1986) *Biochemistry 25*, 7477-7483.
Linden, C. D., Wright, K. L., McConnell, H. M., & Fox, C. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2271-2275.
Mayer, L. M., Hope, M. J., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 858*, 161-168.
Michaelson, D. M., Horwitz, A. F., & Klein, M. P. (1973) *Biochemistry 12*, 2637-2645.
Nordlund, J. R., Schmidt, C. F., & Thompson, T. E. (1981) *Biochemistry 20*, 6415-6420.
Op den Kamp, J. A. F., de Gier, J., & van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 345*, 253-256.
Op den Kamp, J. A. F., Kauerz, M. Th., & van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 169-177.
Papahadjopoulos, D., Jacobsen, K., Niz, S., & Isac, T. (1973) *Biochim. Biophys. Acta 345*, 253-256.
Vail, W. J., & Stollery, J. G. (1979) *Biochim. Biophys. Acta 551*, 74-84.
van Dijck, P. W. M., Ververgaert, P. M. J. T., van Deenen, L. L. M., & de Gier, J. (1975) *Biochim. Biophys. Acta 406*, 465-478.
van Dijck, P. W. M., de Kruijff, B., Verkleij, A. J., van Deenen, L. L. M., & de Gier, J. (1978) *Biochim. Biophys. Acta 512*, 84-96.

Molecular Topography of Toxic Shock Syndrome Toxin 1 As Revealed by Spectroscopic Studies†

Bal Ram Singh, Nighat P. Kokan-Moore,‡ and Merlin S. Bergdoll*
Food Research Institute, University of Wisconsin—Madison, Madison, Wisconsin 53706
Received March 31, 1988; Revised Manuscript Received July 8, 1988

ABSTRACT: Molecular characterization of toxic shock syndrome toxin 1 has been carried out and compared with a group of functionally related staphylococcal enterotoxins. The secondary structure analysis of the far-UV circular dichroic spectrum of toxic shock syndrome toxin 1 revealed 6.25% α-helix, 51.25% β-pleated sheets, 9.0% β-turns, and 33.5% random coils. The pattern, in general, was similar to the staphylococcal enterotoxins. Four antigenic sites have been predicted for toxic shock syndrome toxin 1 by using the secondary structure information in combination with the hydrophilicity calculation. The location of the antigenic sites, in general, agrees with the experimental results. Topographical analysis of the tyrosine residues as determined by second-derivative UV spectroscopy [Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry 23*, 1871-1875] showed that six of nine tyrosine residues are exposed to aqueous solvent. Tryptophan fluorescence quenching studies with an anionic surface quencher, I^-, and a neutral quencher, acrylamide, revealed that almost all of the tryptophan residues are buried in the protein matrix as their accessibility to the surface quencher is very low (17%). Since there are only three tryptophan residues in the amino acid sequence of the toxic shock syndrome toxin 1 and there is a tyrosine residue (Tyr-15, Tyr-115, and Tyr-153) next to each of the tryptophan residues (Trp-14, Trp-116, and Trp-154), it appears the tyrosine residues not exposed to the aqueous solvent are those next to the tryptophan residues. Functional implications of the topography of the tryptophan and tyrosine residues are assessed.

Toxic shock syndrome toxin 1 (TSST-1) is recognized as the major toxin responsible for the signs and symptoms of toxic shock syndrome (TSS) (Bergdoll et al., 1981; Schlievert et al., 1981; de Azavedo & Arbuthnott, 1984; Rasheed et al., 1985). TSS elicits symptoms such as high fever, low blood pressure, rash, hypotension, dizziness, desquamation of the palms of the hands and soles of the feet, and involvement of other organ systems (Todd et al., 1978; Davis et al., 1980; Shands et al., 1980).

TSST-1 is a small molecular weight protein (22K) that has been purified and biochemically characterized (Reiser et al., 1983; Blomster-Hautamaa et al., 1986b). The amino acid sequence shows no significant homology with the staphylococcal enterotoxins, a family of functionally related toxins (Blomster-Hautamaa et al., 1986b; Betley & Mekalanos, 1988), nor does it show any serological cross-reactivity with the enterotoxins. These observations were unexpected in view of the similarity of many of the signs and symptoms produced

† This investigation was supported in part by Procter & Gamble Co., Personal Products of Johnson and Johnson, Kimberly-Clark Corp., Tambrands, Playtex International, Inc., and the College of Agricultural and Life Sciences of the University of Wisconsin-Madison.
* Address correspondence to this author.
‡ Present address: Department of Anatomy and Cellular Biology, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226.

0006-2960/88/0427-8730$01.50/0