Membrane Fusion and Inverted Phases†

Harma Ellens,‡,§ David P. Siegel,∥ Dennis Alford,⊥ Philip L. Yeagle,# Lawrence Boni,△ Leonard J. Lis,◇ Peter J. Quinn,○ and Joe Bentz*,⊥

Department of Pharmacology, School of Medicine, and Departments of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143, Procter and Gamble Company, Post Office Box 39175, Cincinnati, Ohio 45247, The Liposome Company, 1 Research Way, Princeton, New Jersey 08540, Department of Biochemistry, School of Medicine, State University of New York, Buffalo, New York 14214, Department of Physics, Kent State University, Kent, Ohio 44242, and Department of Biochemistry, Kings College London, Kensington Campus, London W87AH, England

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ABSTRACT: We have found a correlation between liposome fusion kinetics and lipid phase behavior for several inverted phase forming lipids. *N*-Methylated dioleoylphosphatidylethanolamine (DOPE-Me), or mixtures of dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC), will form an inverted hexagonal phase (H_{II}) at high temperatures (above T_H), a lamellar phase (L_α) at low temperatures, and an isotropic/inverted cubic phase at intermediate temperatures, which is defined by the appearance of narrow isotropic ^{31}P NMR resonances. The phase behavior has been verified by using high-sensitivity DSC, ^{31}P NMR, freeze–fracture electron microscopy, and X-ray diffraction. The temperature range over which the narrow isotropic resonances occur is defined as ΔT_I, and the range ends at T_H. Extruded liposomes (~0.2 μm in diameter) composed of these lipids show fusion and leakage kinetics which are strongly correlated with the temperatures of these phase transitions. At temperatures below ΔT_I, where the lipid phase is L_α, there is little or no fusion, i.e., mixing of aqueous contents, or leakage. However, as the temperature reaches ΔT_I, there is a rapid increase in both fusion and leakage rates. At temperatures above T_H, the liposomes show aggregation-dependent lysis, as the rapid formation of H_{II} phase precursors disrupts the membranes. We show that the correspondence between the fusion and leakage kinetics and the observed phase behavior is easily rationalized in terms of a recent kinetic theory of L_α/inverted phase transitions. In particular, it is likely that membrane fusion and the L_α/inverted cubic phase transition proceed via a common set of intermembrane intermediates.

Membrane fusion can be analyzed at two levels: (i) kinetic—to ascertain which step of the overall process is rate limiting and what factors influence that step; (ii) structural—to identify the intermembrane structures which either cause fusion or evolve from the fusion intermediate (Bentz & Ellens, 1988). The kinetic analysis of vesicle fusion is now well developed (Nir et al., 1980; Bentz et al., 1983, 1985a, 1988; Ellens et al., 1984). Fluorometric assays for contents mixing, leakage, and the mixing of membrane components have greatly facilitated this work (Düzgüneş & Bentz, 1988).

The structural analysis of liposome fusion began with studies of lipid phase behavior. It was postulated that the intermediates of liposome fusion formed during the course of lipid phase transitions (Suurkuusk et al., 1976; Papahadjopoulos et al., 1977; Verkleij et al., 1980). Subsequent studies found that the observed temperature dependence of liposome fusion did not correlate particularly well with the phase transition tempertures (Düzgüneş et al., 1984; Bentz et al., 1985a,b; Ellens et al., 1986a; Lentz et al., 1987).

There has been some success with correlating liposome fusion kinetics with lateral phase segregation of the lipid constituents (Düzgüneş et al., 1984; Silvius & Gagné, 1984a,b; Leventis et al., 1986; Bentz et al., 1987). This area of study is an important special case of the general problem in that it permits us to understand whether or not the "heterogeneous" membrane might produce local domains of lipids which are fusion competent. However, it does not explain anything more about the fusion intermediate itself than could be determined by using membranes composed of the lipids in the fusion-competent domains. The exception to this would arise if fusion is due to the boundaries between the segregated domains.

Using *N*-methyldioleoylphosphatidylethanolamine (DOPE-Me),[1] Ellens et al. (1986b) found enhanced liposome fusion kinetics in the temperature range in which Gagné et al. (1985) observed isotropic ^{31}P NMR resonances in bulk dispersions

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* Address correspondence to this author at the Department of Pharmacy, University of California, San Francisco.

‡ Department of Pharmacology, University of California, San Francisco.

§ Present address: Drug Delivery Group, Smith, Kline & French Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939.

∥ Procter and Gamble Co.

⊥ Department of Pharmacy and Pharmaceutical Chemistry, University of California, San Francisco.

State University of New York at Buffalo.

△ The Liposome Company.

◇ Kent State University.

○ Kings College London.

[1] Abbreviations: LUV, large unilamellar vesicle(s), ~0.1–0.2-μm diameter; MLV, multilamellar vesicles; SUV, small and sonicated vesicle(s), <0.05-μm diameter; CL, cardiolipin; PA, phosphatidate; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, N-methylated DOPE; EPE, egg phosphatidylethanolamine; TPE, phosphatidylethanolamine prepared from egg phosphatidylcholine by transesterification; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine prepared from TPE; PS, phosphatidylserine; CHEMS, cholesteryl hemisuccinate; ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium bromide); DPA, dipicolinic acid; Tb, terbium Tb^{3+}; T_c, temperature of the lamellar (gel) $L_{\beta'}$-(liquid-crystalline) L_α phase transition; ΔT_I, temperature range from the onset of isotropic ^{31}P NMR resonances to the T_H determined by DSC; T_H, temperature of the L_α-inverted hexagonal H_{II} phase transition; IMI, inverted micelle intermediate; ILA, interlamellar attachment.

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of the same lipid. Because the proper phase of the lipid under these conditions is not known, we refer to the lipid as being in the *isotropic state* at those temperatures where the isotropic resonances are found.[2] When the temperature exceeds the L_α/H_{II} phase transition temperature, T_H, these liposomes undergo a contact-mediated lysis; i.e., the mixing of aqueous contents associated with fusion is abolished, as has been found for many other systems (Bentz et al., 1985b, 1987; Ellens et al., 1986a,b). Above T_H, there is also a prodigious amount of lipid mixing as the liposomal membranes are transformed to H_{II} phase precursors and the bilayers cease to exist.

We would like to understand the intermembrane structures which produce these isotropic resonances, since they are putative fusion structures. We know that the isotropic resonances are due to a fraction of the phospholipids in the system experiencing isotropic motional averaging on the ^{31}P NMR time scale [ca. 20 μs (Campbell et al., 1979; Larsen et al., 1987)]. They appear under the same circumstances as lipidic particle morphology, observed by electron microscopy [e.g., see de Kruijff et al. (1979), Gagné et al. (1985), Boni and Hui (1983), and Hui et al. (1983)], and as amorphous and inverted cubic phases, observed by X-ray diffraction [e.g., see Rilfors et al. (1982, 1986), Boni and Hui (1983), Hui et al. (1983), Kirk (1984), and Gruner et al. (1988)]. However, there is no definitive answer as to the structure of these intermediates (Hui et al., 1981, 1983; Boni & Hui, 1983; Verkleij, 1984; Borovagin et al., 1982; Cullis et al., 1985).

Recent theoretical studies on the mechanism of L_α/H_{II} phase transitions postulated a sequence of intermembrane structures which can account for most of the NMR, DSC, and morphological data concerning these transitions (Siegel, 1986a–c, 1987; Siegel et al., 1988). The postulated intermediates are consistent with models for the relative chemical potentials of inverted phase structures (Kirk et al., 1984; Gruner, 1985, 1986), as well as with results of X-ray diffraction studies of these systems (Caffrey, 1985, 1987; Tate & Gruner, 1987; Gruner et al., 1988). These theoretical studies also predict that a subset of systems (including DOPE-Me and PE/PC mixtures) with L_α/H_{II} transitions should form a type of intermediate that would produce isotropic ^{31}P NMR resonances in a temperature interval starting tens of degrees below T_H (Siegel, 1986c).

Here we have studied the fusion and leakage of liposomes composed of DOPE-Me and of several DOPE/DOPC mixtures, as well as the equilibrium phase behavior of these systems using high-sensitivity DSC, ^{31}P NMR, time-resolved X-ray diffraction, and rapid freeze–fracture electron microscopy. We conclude that liposome fusion, isotropic ^{31}P NMR resonances, and inverted cubic lattice formation share a common molecular intermediate. Of course, this mechanism of fusion is relevant only in lipid systems that have a nearby H_{II} phase boundary, and then only if isotropic ^{31}P NMR resonances are observed on the first heating scan (Ellens et al., 1986b; Siegel, 1986b,c; Siegel et al., 1988). The current status of other liposome fusion mechanisms has been discussed elsewhere (Bentz & Ellens, 1988; Bentz et al., 1988).

Materials and Methods

Dioleoylphosphatidylethanolamine (DOPE), DOPE with a single methyl substitution at the amino group (DOPE-Me), dioleoylphosphatidylcholine (DOPC), and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE prepared from transesterified egg PC (NBD-PE) were purchased from Avanti Polar lipids (Birmingham, AL). 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX) were from Molecular Probes Inc. (Junction City, OR). Terbium chloride ($TbCl_3$) was from Aesar Rare Earth Products (Seabrook, NH), nitrilotriacetic acid (NTA) was from Aldrich (Milwaukee, WI), and dipicolinic acid (DPA) was from Sigma.

Liposome Preparation. The liposomes were prepared according to Szoka and Papahadjopoulos (1978). Further details on all these procedures can be found in Ellens et al. (1985, 1986a,b) and Bentz et al. (1988). For the ANTS/DPX leakage and fusion assay (Ellens et al., 1985), the liposomes contained either (i) 25 mM ANTS and 45 mM NaCl, (ii) 90 mM DPX, or (iii) 12.5 mM ANTS, 45 mM DPX, and 22.5 mM NaCl. For some experiments, the Tb/DPA assay was used (Wilschut et al., 1980). In these cases, the liposomes contained either (i) 2.5 mM $TbCl_3$, 50 mM NTA, and 20 mM NaCl, (ii) 50 mM DPA, or (iii) 1.25 mM $TbCl_3$, 25 mM NTA, 10 mM NaCl, and 25 mM DPA. All solutions were buffered with 10 mM glycine at pH 9.5. In all cases, the encapsulated solutions were isoosmotic to the buffers used for the column chromatography and in the leakage and fusion experiments. To measure the change in NBD fluorescence quantum efficiency, liposomes composed of DOPE-Me or DOPE/DOPC containing 0.1 mol % of NBD-PE were made in the isoosmotic NaCl/glycine buffer. The liposomes were extruded through polycarbonate membranes (Nuclepore Corp., Pleasanton, CA) with 0.1-μm pores and separated from unencapsulated material on Sephadex G-75 (Pharmacia) using 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA (pH 9.5) for the elution buffer. There was no significant binding of ANTS to the exterior of the liposomes (Ellens et al., 1984, 1985, 1986a).

The size distribution of the liposomes was measured using dynamic light scattering (Coulter Model N4; Coulter Electronics, Inc., Hialeah, FL). For the DOPE-Me liposomes used in the fusion studies, which were extruded once, the *Z*-average diameter was 270 ± 95 nm for the ANTS liposomes, 220 ± 75 nm for the ANTS/DPX liposomes, and 280 ± 100 nm for the DPX liposomes. The encapsulated volume was 3.2 μL/μmol of lipid for the ANTS/DPX-containing liposomes and 5 μL/μmol of lipid for the ANTS-containing liposomes. Since the area per DOPE-Me headgroup in the bilayer is about 60 Å^2 (Mulukutla & Shipley, 1984; Gruner et al., 1988), we know that the liposomes have two or more lamella. For the DOPE/DOPC (3:1) liposomes, which are extruded at least 4 times, smaller diameters were found, 170 ± 45 nm, and the encapsulated volumes were 2.3 ± 0.1 μL/μmol, for all of the encapsulated fluorophores of both assays. The repeated extrusion appears to produce more uniform liposomes, but they still must be oligolamellar.

The absolute rates of leakage and fusion varied between liposome preparations, but the temperature dependence of the kinetics was the same for all liposome preparations. The encapsulated contents of liposomes stored at pH 9.5 and 4 °C showed no measurable leakage for at least a week. All samples were used within 2 days.

Fusion Studies. Fluorescence and light scattering were measured with either a Spex Fluorolog 2 (Spex Industries, Edison, NJ) or an SLM 4000 fluorometer (SLM Instruments, Champaign—Urbana, IL) equipped with two 90° emission channels, allowing both fluorescence and light scattering to

[2] Throughout this work, the terms isotropic state and ΔT_I are defined operationally by the presence of the narrow isotropic ^{31}P NMR resonances and the temperature range over which these resonances are observed, respectively. For some lipid systems, these resonances are associated cubic-phase X-ray diffraction patterns (Luzzati & Reiss-Husson, 1966; Rilfors et al., 1986; Gruner et al., 1988; Siegel et al., 1989b).

be monitored simultaneously. With the ANTS/DPX fusion assay, mixing of aqueous contents of ANTS- and DPX-containing liposomes is registered as a decrease in ANTS fluorescence due to quenching of ANTS by DPX (Ellens et al., 1985). Leakage is measured with liposomes containing both ANTS and DPX (Ellens et al., 1984). The fluorescence scale was calibrated with the intensity of the mixture of ANTS and DPX liposomes in glycine buffer (100 mM NaCl, 2 mM glycine, and 0.1 mM EDTA, pH 9.5) taken as 100% fluorescence (0% fusion). The 0% fluorescence level was set to the intensity of the liposomes containing the coencapsulated ANTS/DPX, i.e., the leakage liposomes. Excitation was at 360 nm and emission >530 nm. On the figures, fusion (percent maximum Q) is equal to 100 minus the recorded fluorescence, which is equal to the percentage of ANTS which is quenched by DPX at that time. ANTS fluorescence is not sensitive to pH between 9.5 and 4.5, and the quenching efficiency of DPX is not affected by pH in this region.

The normal procedure for calibrating the 100% fluorescence intensity level for the Tb/DPA fusion assay (Wilschut et al., 1980; Düzgüneş & Bentz, 1988) could not be used at high temperatures. Briefly, this procedure calls for lysing (e.g., 50 μM lipid) of Tb-containing liposomes (type i above) in an EDTA-free buffer containing 20 μM free DPA, which is excess DPA at 20 °C. At room temperature, this fluorescence intensity is equal to that of 100 μM (lipid) Tb/DPA-containing liposomes (type iii above), since the encapsulation volumes are essentially identical. As the temperature is increased, the absolute intensity of these two standards decreased, and most importantly, the intensity of the free Tb/DPA solution decreased far more rapidly than did that of the liposome-encapsulated Tb/DPA, especially above 50 °C. Part of this decrease may reflect a diminished quantum efficiency. However, it is very likely that the *difference* between the free solution and the liposome-encapsulated Tb/DPA is due to dilutional dissociation of the Tb/DPA complex in the free solution. Lysing the liposomes dilutes the Tb concentration about 4000 times. The simple mass action complexation may be expected to show increased dissociation as the temperature increases.

Since the nonleaky fusion of a Tb-containing and a DPA-containing liposome would encapsulate the same solution as is in the Tb/DPA-containing liposomes, we have chosen to calibrate the 100% levels using the Tb/DPA-containing liposomes, equilibrated to the appropriate temperature. The alternative approach of increasing the DPA concentration in the free solution with the lysed Tb-containing liposomes is untenable due to excess light adsorption by the DPA. Using the free solutions for calibration would certainly overestimate the extent of contents mixing since the "100%" would be set artificially low.

The fusion was started by injection of an aliquot of the various stock solutions into a magnetically stirred cuvette, containing 2.0 mL of the liposome suspension in glycine buffer (100 mM NaCl, 2 mM glycine, and 0.1 mM EDTA, pH 9.5). To achieve pH 7.4, the aliquot was 20–30 μL (depending upon the temperature) of a 1.0 M TES/pH 7.0 buffer. To achieve pH 7.4 with 5 mM Mg^{2+}, the aliquot was 25 μL of a 0.4 M $MgCl_2$/1.0 M TES/pH 7.0 buffer. To achieve pH 9.5 with 20 mM Mg^{2+}, the aliquot was 25 μL of a 1.60 M $MgCl_2$/pH 6.8 buffer. To achieve pH 4.5, the aliquot was 20 μL of a 5 M acetic acid/acetate/pH 4.5 buffer. All final pH values were measured.

Differential Scanning Calorimetry. To ensure full and uniform hydration, samples for DSC, NMR, and X-ray diffraction studies were all made according to the following protocol. In two-component systems, either lipids were weighed out as powders and cosolubilized in chloroform or the chloroform solutions of the component lipids were mixed. Lipid concentrations in multicomponent systems are accurate to ±2%. Chloroform was removed via rotary evaporation for 30 min at room temperature under high vacuum. The lipid was hydrated (<6 mg/mL) for 2–3 h at 4 °C, extensively vortexed, and then subjected to three freeze–thaw cycles (dry ice and a water bath at 50 °C, respectively). The lipid was then pelleted for 20 min in an ultracentrifuge (25 000 rpm in an SW-40 Ti rotor; 80000g) at 4 °C and resuspended at the final concentration. If the buffer employed contained divalent cations, the sample was pelleted and resuspended in a new 10-mL aliquot of buffer after each freeze–thaw cycle. This procedure yielded the same DSC results as samples dialyzed in excess buffer for over 24 h. If the sample made up in this manner was frozen for later use (at −70 °C, as occasionally occurred prior to NMR or X-ray experiments), the samples were subjected to three freeze–thaw cycles just prior to examination. Samples not treated in this manner occasionally exhibited isotropic ^{31}P NMR resonances at lower temperatures than samples prepared immediately before use. This type of hysteresis has been noted previously (Gruner et al., 1988).

The buffers were all 100 mM in NaCl, containing in addition either (i) 50 mM acetate/0.1 mM EDTA at pH 4.5; (ii) 20 mM $MgCl_2$, 10 mM glycine at pH 9.5 or 9.9, and 0.1 mM EDTA; or (iii) 5 mM $MgCl_2$, 10 mM TES at pH 7.4, and 0.1 mM EDTA. Thermograms were obtained by using a Microcal MC-2 calorimeter at a scan rate of either 13 or 27 °C/h. There was no significant difference in the onset or peak temperatures of the L_α/H_{II} phase transition between thermograms obtained at these two scan rates. For DOPE-Me at neutral or low pH, we found a small low-temperature shoulder to the transition in most, but not all, of the thermograms when the scan rate was 13 °C/h. The significance of this shoulder is discussed below.

X-ray Diffraction. Samples were prepared and equilibrated as above. X-ray diffraction patterns were obtained at station 7.3 of the synchrotron radiation source at the SERC Daresbury Laboratory, using 0.150-nm radiation and a linear detector constructed at the Daresbury Laboratory, as described previously (Nave et al., 1985; Tenchov et al., 1987). The samples were placed between mica windows in a brass chamber thermostated via an external circulating water bath. The temperature of the sample cell was continuously monitored via a thermocouple. The temperature of the sample cell was increased in an approximately linear fashion at ca. 9 °C/min as a total of 255 consecutive diffraction patterns were obtained, each pattern being compiled in 1.2 s with a dead time of 50 μs between frames. The acquired data were stored in a VAX-11/750 computer and corrected for detector response by comparison with a pattern recorded using a fixed source and averaged over several hours. Data were smoothed with the OTOKO program, which uses a cubic spline interpolation subroutine. Static X-ray patterns were obtained at constant temperature using a Rigaku RU-100H rotating-anode X-ray source and a flat-film camera.

^{31}P NMR. Samples were prepared and equilibrated as above. ^{31}P nuclear magnetic resonance (NMR) spectra were obtained as described previously (Yeagle & Sen, 1986) with a JEOL FX270 Fourier-transform spectrometer with a broad-band probe in 10-mm tubes. A total of 2000 scans were collected with a repetition rate of 1 s. A fully phased cycled (32 pulse) Hahn echo sequence was used with a 40-μs echo.

The echo sequence eliminates base-line artifacts, removing the need for first-order phase corrections (Rance & Bird, 1983). Data were collected prior to the refocusing of the echo and the FID transformed from the top of the echo. This procedure avoids artifacts that arise when the receiver is turned on only at the expected refocusing of the echo. Because of the short echo time that must be employed to capture all the resonance intensity (due to the very short T_2*), the finite length of the transmitter pulses ($\pi/2$ = 10 μs) must be taken into account in determining the refocusing point of the echo. Because of the very rapid decay in the FID of the broad component in these spectra, care must be taken to transform from the top of the echo because the broad features are readily lost in the Fourier transform if the refocusing point of the echo is missed. The ^{1}H decoupler was gated on during acquisition and off the remainder of the time to prevent sample heating. The effectiveness of the decoupling was improved by using single-frequency decoupling, rather than noise-modulated decoupling, with the frequency set at the resonance frequency of the phospholipid headgroup protons. Exponential line broadening of 50–200 Hz was used. Recalculations showed no introduction of artifacts occurred in this range.

Freeze–Fracture Electron Microscopy. DOPE–Me (50 μmol) and NBD-PE (0.05 μmol) were mixed in chloroform and dried down in a rotary evaporator for 1 h under high vacuum. The lipid was hydrated in 10 mL of glycine buffer (10 mM glycine/100 mM NaCl/pH 9.5) by brief sonication and vortexing, followed by one freeze–thaw cycle. The micrographs of the MLV were of these liposomes. For the LUV, a portion of these liposomes were then extruded through a 0.4-μm filter and 4 times through a 0.2-μm filter at 120 psi (Hope et al., 1985).

To lower the pH, 75 μL of 2 M acetate/acetic acid stock (pH 4.5) was injected into 3 mL of a 200 μM liposome suspension at 55 °C. To achieve 20 mM Mg^{2+}/pH 9.5 (at 45 °C), 60 μL of 1.0 M $MgCl_2$/pH 6.8 stock was injected into the cuvette. The NBD fluorescence and 90° light scattering were followed until the LUV suspension showed "collapse" (~2 min), as described in Figure 4. The MLV suspension showed an increase in the intensity of scattered light, which was followed by a decrease in intensity, perhaps due to massive aggregation. Ten minutes after the time of LUV collapse, both LUV and MLV samples were cooled to room temperature for freeze–fracture.

Samples for freeze–fracture were taken directly from the cuvettes. A 0.1–0.3-μL aliquot, without cryoprotectant, was sandwiched between a pair of Balzers (Nashua, NH) copper support plates and rapidly plunged into liquid propane (Costello & Corliss, 1978). The specimen was fractured and replicated in a Balzers BAF 400 freeze–fracture unit at a vacuum of 3×10^{-7} mbar, or better, and at −115 °C. Replicas were floated off in HNO_3 (3.0 N) and washed in a graded series of Clorox solutions. Replicas were viewed on a Philips 300 electron microscope at magnifications of 6000–75000×.

Results

Structural Studies: DSC, X-ray Diffraction, and ^{31}P NMR. The onset and peak L_α/H_{II} transition temperatures for all the systems studied are given in Table I. The peak and onset temperatures were generally reproducible to within 2 °C or better, although DOPE/DOPC (3:1) in the presence of 20 mM Mg^{2+} at pH 9.5 yielded less reproducible temperatures for unknown reasons. Endotherms obtained at scan rates of 13 and 27 °C/h were the same within experimental error. For the DOPE-Me systems, the endotherms were relatively narrow, with full widths at half-height (FWHH) of 1.0–1.3 °C and transition enthalpies of ca. 200 cal/mol. For the DOPE/DOPC systems, the endotherms were more poorly defined, with FWHH of 3–5 °C and enthalpies of ca. 70 cal/mol for DOPE/DOPC (3:1) systems and FWHH at 4–8 °C and ca. 100 cal/mol for DOPE/DOPC (4:1) systems. The endotherms of the (4:1) systems were often skewed to the high-temperature side. Endotherms in the DOPE/DOPC (2:1) system were of very low amplitude, and the transition temperatures were poorly reproducible, ranging from 76 to 83 °C in peak temperature.

Table I: L_α–H_{II} Phase Transition Temperatures As Determined by DSC

lipid system	cations (all buffers contain 100 mM NaCl)	transition temp (°C) onset ± SD	transition temp (°C) peak ± SD
DOPE-Me	pH 4.5	64 ± 2	66 ± 2
	pH 7.4[a]	65 ± 2	66 ± 2
	pH 9.5/20 mM Mg^{2+}	58 ± 2	60 ± 2
	pH 7.4/5 mM Mg^{2+}	65 ± 2	66 ± 2
DOPE/DOPC (2:1)	pH 4.5	≥80[b]	
	pH 9.5/20 mM Mg^{2+}	≥80[b]	
DOPE/DOPC (3:1)	pH 4.5	56 ± 3	60 ± 4
	pH 9.5/20 mM Mg^{2+}	67 ± 4	71 ± 5
	pH 7.4/5 mM Mg^{2+}	59 ± 4	62 ± 4
DOPE/DOPC (4:1)	pH 4.5	43 ± 2	47 ± 2
	pH 9.5/20 mM Mg^{2+}	54 ± 2	56 ± 2

[a] The transition temperatures are the same if the buffer contains 150 mM NaCl. [b] Ill-defined small endotherm with an onset temperature of ≥80 °C.

Peak temperatures from the same lot of lipid were reproducible to within 1.0 °C. Different lots occasionally yielded transition temperatures which were as much as 3 °C different, although 2D TLC indicated no difference in purity (to 0.5 mol % accuracy). Most of the variability between the PE/PC mixtures can be ascribed to small variations in the PE mole fraction, where a 1% change can change T_H by 2–3 °C. The data cited here are averaged over several lot numbers.

The widths of the DOPE/DOPC system endotherms indicate that the L_α and H_{II} phases coexist over substantial temperature ranges. For the sake of simplicity, we will use the peak temperature of an endotherm as the value of T_H. However, the onset temperatures in Table I (obtained by extrapolation of the low-temperature side of the endotherm to the extrapolated base line) are more representative of the temperatures at which the H_{II} phase can begin to form in given systems.

T_H increases as the DOPE/DOPC mixtures contain more DOPC, as shown by Tilcock et al. (1982). For these mixtures, the dependence of T_H on PC mole fraction is roughly linear. For pure DOPE, $T_H \simeq$ 6–10 °C (Epand, 1985; Gagné et al., 1985).

Clearly, either cation (H^+ or Mg^{2+}) can neutralize the membrane surfaces sufficiently to permit the transition. H^+ binds to the amino group, yielding a zwitterionic headgroup [pK_a ~ 9.5 for DPPE in DPPC SUV; (Tsui et al., 1986)]. Mg^{2+} presumably binds to the phosphate (McLaughlin et al., 1981; Lau et al., 1981). It is interesting that for DOPE-Me, the T_H is lower with Mg^{2+} than with H^+, whereas the opposite result is found with the DOPE/DOPC mixtures. This effect should be useful for elucidating the role and position of cation binding on the thermodynamics of the phase transition. On the other hand, the T_H is essentially the same for DOPE-Me at pH 4.5 and at pH 7.4 with 5 mM Mg^{2+} in the medium (Table I). Previously, we found that pH 4.5 and 7.4 gave essentially identical T_H's (Ellens et al., 1986b); thus, the presence of 5 mM Mg^{2+} at pH 7.4 has little effect upon T_H.

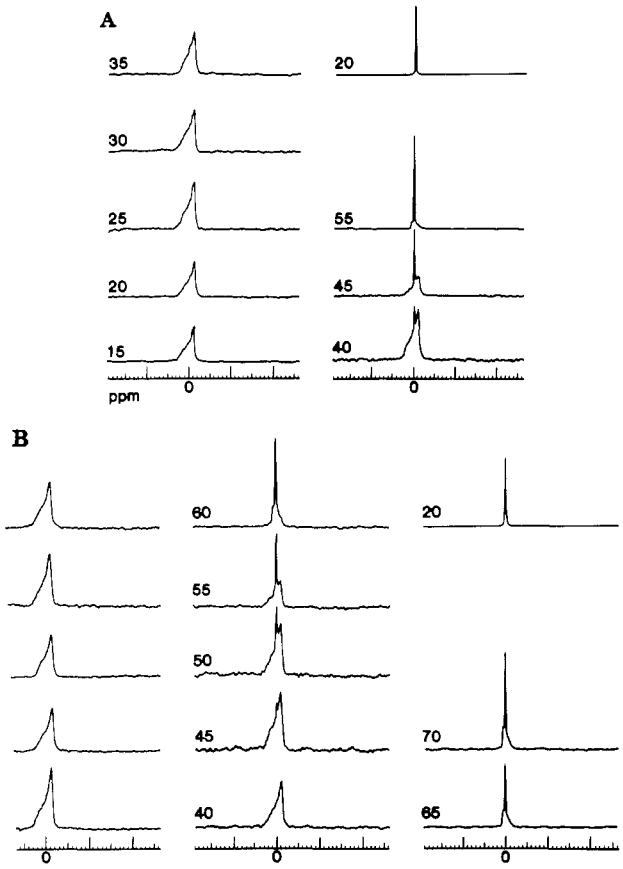

FIGURE 1: ^{31}P NMR spectra are shown for DOPE-Me at pH 9.5 in the presence of 20 mM Mg^{2+} (A) and for DOPE/DOPC (3:1) at pH 7.4 in the presence of 5 mM Mg^{2+} (B). The spectra were obtained by scanning from lower to higher temperatures, except the top right spectra which were obtained by cooling the same sample back down to 20 °C. Interestingly, in all cases, the signal after cooling back to 20 °C is completely isotropic. Major divisions of the indicated scale of chemical shifts represent 100 ppm.

We obtained time-resolved X-ray diffraction patterns for several of the systems studied calorimetrically. This was done to verify that the observed transitions were to the H_{II} phase.

The samples studied were DOPE-Me at pH 4.5 and 7.4 and with 20 mM Mg/pH 9.5 and DOPE/DOPC (3:1) at pH 4.5 and with 5 mM Mg/pH 7.4. The diffraction patterns indicate the existence of no lattices other than L_α and H_{II}. Of course, this does not rule out the formation of short-lived or randomly dispersed structures, neither of which would provide coherent reflections. There are several reports of inverted cubic phase X-ray diffraction patterns being observed in similar systems, but only after long time periods (Gruner et al., 1988; Shyamsunder et al., 1988; Siegel et al., 1989b). Thus, we would not expect these patterns to arise during the rapid scans (9 °C/min) used in this study. We noted that upon cooling certain samples below T_H, the diffraction patterns showed that the sample did not return to a well-ordered L_α phase, as had been found also by Gruner et al. (1988).

^{31}P NMR indicates the presence of additional structures. Figure 1 shows spectra for DOPE–Me with 20 mM Mg/pH 9.5 (A) and DOPE/DOPC (3:1) with 5 mM Mg/pH 7.4 (B). At the lower temperatures in each sample, the NMR spectra show the axially symmetric powder pattern characteristic of phospholipids in L_α phases (Cullis & Hope, 1978). The most striking feature of these spectra is the emergence of an isotropic resonance at 35 °C for DOPE-Me and at 45 °C for DOPE/DOPC (3:1), 25 and 12 °C below the T_H of the systems, respectively. It is possible that these resonances mark the first step in the formation of an inverted cubic phase, such as that reported in DOPE-Me at neutral pH (Gruner et al., 1988; Siegel et al., 1989b). Formation of this phase is often not evident in DSC data because of the long incubation times necessary for the appearance of macroscopic amounts of it and because of the trivial enthalpies ($\ll kT$) found in samples at the lipid concentrations used (Siegel, 1986c; Gruner et al., 1988; Siegel et al., 1989b). In both of the systems in Figure 1, a ^{31}P NMR powder pattern characteristic of the H_{II} phase emerges (Cullis & Hope, 1978) at temperatures close to the L_α/H_{II} transition temperature determined via DSC, i.e., at ca. 55 °C in (A) and 60 °C in (B), as compared to DSC transition peak temperatures of 60 and 62 °C, respectively (Table I).

When the hexagonal phase samples were cooled back to 20 °C, the result is a pure isotropic resonance; i.e., the samples do not return to a well-ordered L_α phase. It has been noted previously that repeated freeze–thaw cycles are required to reestablish long-range order in the L_α phase (Cullis et al., 1978b; Ellens et al., 1986b; Gruner et al., 1988).

The ^{31}P NMR resonance is extremely sensitive with respect to the amount of lipid which is involved in isotropic motion. By measuring the area under the curves, we can make a rough estimate of these percentages. Because of the narrowness of the ^{31}P NMR resonances, it is possible to detect such components when they arise from as little as a few percent of the total phospholipid. In Table II, we have collected the onset temperatures (T_I) for isotropic ^{31}P NMR resonances for the lipid systems. For example, Figure 1A gives T_I = 35–40 °C for DOPE-Me with 20 mM Mg/pH 9.5.[3]

Fusion Studies. Liposomes were made by the REV procedure and extruded through 0.1-μm Nucleopore filters (Ellens et al., 1986a,b). Fusion was monitored by the Tb/DPA or the ANTS/DPX assay for the mixing of encapsulated contents within the fused liposomes. Figure 2A shows the fusion of DOPE/DOPC (3:1) liposomes induced with 5 mM Mg^{2+} at pH 7.4, using the Tb/DPA assay. Figure 2B shows the decrease of fluorescence intensity due to the dissociation of preencapsulated Tb/DPA complex. This decrease is due to both the leakage of contents and the influx of medium into the fusing liposomes (Bentz et al., 1985a; Düzgüneş & Bentz, 1988). In both instances, the Tb/DPA complex is disrupted by EDTA and Mg^{2+} in the medium. In this case, there is little

Table II: Onset Temperatures of Isotropic ^{31}P NMR Resonances[a]

lipid	cations[b]	T_I (°C)
DOPE-Me	pH 4.5	40–45[c]
	pH 7.4	30–35,[d] 40–50[c]
	5 mM Mg/pH 7.4	45–50[c]
	20 mM Mg/pH 9.5	35–40[c]
DOPE/DOPC (2:1)	pH 7.4	65–70[f]
DOPE/DOPC (3:1)	5 mM Mg/pH 7.4	45–50[c]
DOPE/DOPC (4:1)	pH 7.0	<35[e]

[a] These temperatures are not phase transition temperatures and can be very sensitive to equilibration techniques and contamination. Data produced in this study did give reproducible ΔT_I values.[3] [b] The media also contain 0.1–0.2 M NaCl unless otherwise noted, and the sample is in excess water. [c] This work. [d] Gagné et al. (1985). [e] Tilcock et al. (1982). [f] van Duijn et al. (1986).

[3] Tilcock et al. (1982) found that the isotropic resonance intensity increased markedly in the first 30 min, following a 10-min equilibration. However, Boni and Hui (1983) did not observe any changes in the intensity of the isotropic resonance over the course of 6 h. Likewise, for the data presented here, there was no evidence of time evolution. In several cases, a second ^{31}P NMR scan was taken at the same temperature (each one requiring about 15 min), and we could find no significant difference between them. It is clear that a rigorous equilibration routine is crucial for these types of experiments. Gruner et al. (1988) reached the same conclusion.

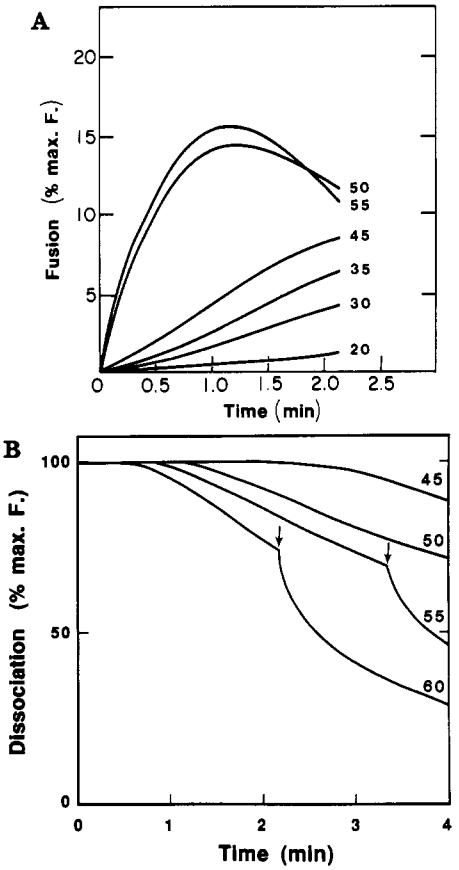

FIGURE 2: Temperature dependence of the fusion and leakage of DOPE/DOPC (3:1) liposomes at pH 7.4 with 5 mM Mg^{2+}. Panel A shows the fusion measured using the Tb/DPA assay for mixing of aqueous contents. The temperature is shown beside each curve in degrees centigrade. Panel B shows the loss of fluorescence intensity of preencapsulated Tb/DPA complex due to the leakage of contents and the influx of medium following the fusion of these liposomes. The Tb/DPA complex is dissociated by the competitive chelation of Tb^{3+} by EDTA and of DPA by Mg^{2+}. The arrows in panel B mark the collapse event, a sharp ejection of liposomal contents, which is described in detail in Figures 4–6, where the arrows also mark the collapse in those figures.

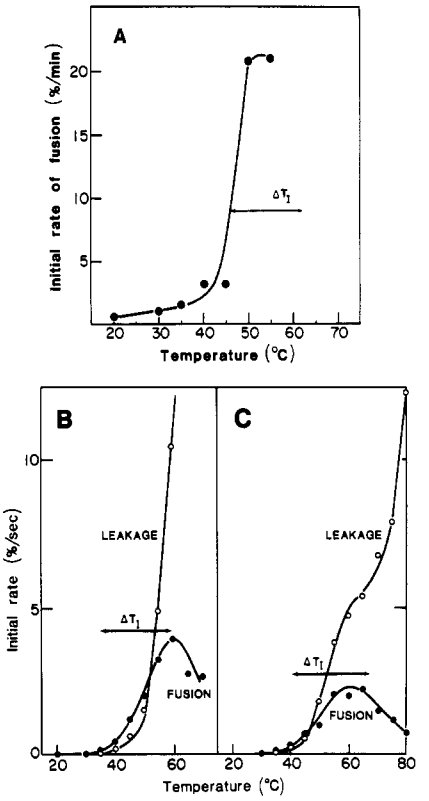

FIGURE 3: Initial rates of fusion of DOPE/DOPC (3:1) liposomes in 5 mM Mg^{2+}/pH 7.4 (A) and of DOPE-Me liposomes in 20 mM Mg/pH 9.5 (B) and at pH 4.5 (C). The initial rate is the initial slope of the fusion curve, as in Figure 2A. The temperature range of the isotropic ^{31}P NMR resonances is demarked by ΔT_I, which ends at the DSC-measured hexagonal phase transition temperature, T_H. Fusion is promoted in the temperature range of the isotropic ^{31}P NMR signal. Fusion for the DOPE-Me liposomes was measured by using the ANTS/DPX contents mixing assay.

leakage initially. However, over time, the leakage increases, especially above 45 °C which is the beginning of ΔT_I. The ANTS/DPX assay system gave the same qualitative results, except that leakage is more rapid initially. We believe this difference is due to the liposome preparations, rather than some discrepancy between the assays. The absolute rates of fusion and leakage varied between liposome preparations, even when the same assay system was used. For DOPE-Me liposomes containing 2 mol % diacylglycerol in pH 7.4/5 mM Mg^{2+}, both assays showed qualitatively the same behavior [see Siegel et al., 1989a)]. A detailed comparison of the two assays when encapsulated in the same liposome is currently in progress (Alford et al., 1989).

Often at the higher temperatures, there is an eventual abrupt loss of fluorescence due to an ejection of contents following "collapse" of the fusing structures. We will describe this event in fuller detail in the next section.

In Figure 3, the initial rate of fusion (i.e., the initial slope of the fluorescence curves) is plotted versus temperature for three systems. The temperature ranges where isotropic ^{31}P NMR resonances are found, ΔT_I, are shown (Table II).

For DOPE/DOPC (2:1), similar results have been found previously. At pH 4.5, there is a sharp increase in the fusion rate at ~60 °C and a decrease in the rate at ~80 °C (Ellens et al., 1986b). Subsequently, van Duijn et al. (1986) found at pH 7.4 that ΔT_I begins at ~60 °C, using ^{31}P NMR. Their NMR data also showed no H_{II} phase resonances up to 70 °C. ΔT_I for DOPE-Me is insensitive to pH in the range of 4.5–7.4 (Table II). To the extent that this is also the case for DOPE/DOPC (2:1), these data confirm the speculation in Ellens et al. (1986b) that the increase in fusion kinetics at 60 °C was correlated with the onset of the isotropic resonances. These liposomes also showed severalfold slower fusion from 40 to 60 °C, where the equilibrium phase is L_α. As discussed below, we believe that this fusion is due to another mechanism.

For all of these systems, it is obvious that when the temperature reaches ΔT_I, there is a sharp increase in the rate of fusion and in the rate of leakage. When the temperature reaches T_H, the liposomes undergo contact-mediated lysis, and fusion is abolished (Ellens et al., 1986a,b; Bentz et al., 1987).

Collapse following Fusion. In Ellens et al. (1986b), we described a unique phenomenon associated with the fusion of DOPE-Me liposomes. After a certain time period of fusion, there is an abrupt ejection of the liposomal contents into the medium, which we refer to as collapse. Figure 2B shows this abrupt leakage with DOPE/DOPC (3:1) liposomes fusing in 5 mM Mg^{2+} at pH 7.4, as monitored by the dissociation of preencapsulated Tb/DPA complex. While we define the event by the abrupt ejection of aqueous contents, we have found that the scattered light intensity abruptly changes at the same time. In those instances where collapse occurs after significant gradual leakage, the abrupt change in the light scattering is the clearest indication that collapse has occurred.

The collapse phenomenon can also abruptly change the quantum yield of fluorescently labeled lipids. Figure 4 shows

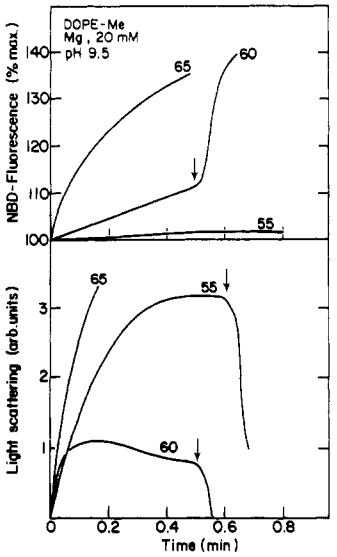

FIGURE 4: Time course of NBD fluorescence increase in DOPE-Me liposomes with 20 mM Mg/pH 9.5 at various temperatures. The upper panel shows the fluorescence curves, and the lower panel shows the corresponding 90° light-scattering traces. It is clear that the collapse, at higher temperatures, can signal a rapid increase in NBD fluorescence. The fluorescence scale is set with buffer at 0% and the initial fluorescence (pH 9.5 without Mg^{2+}) at 100%. The arrows mark the onset of the collapse event.

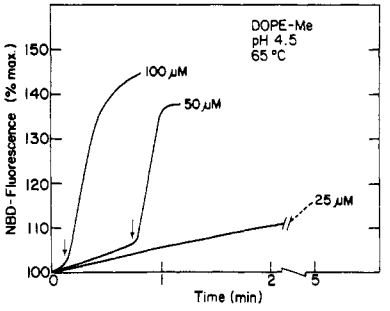

FIGURE 5: Effect of lipid concentration on the collapse time as monitored by NBD fluorescence. The arrows mark the onset of the collapse event.

this event using DOPE-Me liposomes with 20 mM Mg/pH 9.5. Here we monitored the 90° light scattering (lower panel) and the fluorescence of NBD-PE incorporated into the liposomes at 0.1 mol %, i.e., where there is no energy transfer or self-quenching (Struck et al., 1981). Under these conditions, the NBD quantum efficiency can be used to monitor the transition to the H_{II} phase (Ellens et al., 1986a,b; Bentz et al., 1987; Hong et al., 1988). At 65 °C ($>T_H$), the NBD quantum efficiency immediately increases (upper panel), as the liposomes begin transforming to H_{II} phase precursors upon contact (Ellens et al., 1986a,b). At 60 °C ($\sim T_H$), there is a slow increase in fluorescence until ~0.5 min when the collapse demarks a rapid transition to inverted phase aggregates. There is a simultaneous abrupt decrease in the 90° light-scattering intensity. There is also a loss of aqueous contents (data not shown). At 55 °C ($<T_H$), we see no significant change in NBD fluorescence after the 90° light scattering demarks the collapse.

We have found that collapse occurs after a considerable extent of aggregation/fusion has occurred. However, Figure 5 indicates that collapse may not be simply a matter of the aggregation/fusion products reaching a particular size. NBD fluorescence from DOPE-Me liposomes shows that collapse occurs faster with 100 μM lipid than with 50 μM lipid, as an aggregation-dependent event would show. However, with 25

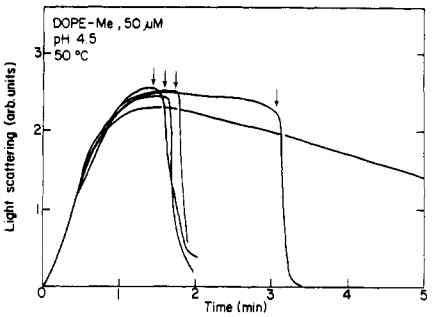

FIGURE 6: Ninety degree light scattering monitors the collapse of 50 μM DOPE-Me liposomes at pH 4.5 and 50 °C. The same experiment is repeated 5 times. There is a great deal of variability in the time at which collapse occurs. The arrows mark the onset of the collapse event.

μM lipid, the collapse did not occur even after 5 min. Simple kinetic explanations, such as a delay in the time of collapse due to the effect of aggregation reversibility at low lipid concentrations (Bentz & Nir, 1981; Ellens et al., 1984), are not very compelling under these circumstances (Bentz et al., 1988).

Collapse is a rather hysteretic event. Figure 6 shows the 90° light scattering for DOPE-Me liposomes at pH 4.5 and 50 °C. Here we repeated the experiment 5 times under identical conditions. In three cases, collapse occurred at about the same time (1.5–2 min). In one case, the collapse took about twice as long (~3 min). Finally, in one case, there was no collapse after 5 min. The fact that the light-scattering intensities were initially identical is clear evidence that the hysteresis is not due to errors in sample mixing.

Electron Microscopy. We have examined the products of collapse using freeze-fracture electron microscopy on DOPE-Me liposomes. Figure 7 shows these results. The upper panels show products of LUV (~0.15-μm diameter, initially) taken 10 min after collapse due to incubation at pH 4.5 and 55 °C (panel a) and with 20 μM Mg/pH 9.5 and 45 °C (panel b). For comparison, we show the corresponding products when the initial liposome samples are handshaken MLV (~0.3–1.0-μm diameter, initially). The light scattering for the MLV increases and then decreases in intensity, due to extensive aggregation, but there is no evidence of the abrupt change we define as collapse. Panel c shows the MLV at pH 4.5, and panel d shows the effect of 20 mM Mg/pH 9.5. The bar indicates 150 nm. The MLV do show evidence of some lipidic particle formation, although there are large areas of lamellar structure. The lipidic particles are far more numerous in the LUV samples. Most importantly, there are large domains of well-ordered lipidic particles, which have been correlated with cubic phase formation (Hui et al., 1983; Boni & Hui, 1983; Rilfors et al., 1986). We also note that the dimpled surfaces appear to show a few bilayers covering the domains of aligned lipidic particles.

The differences in the morphology between the LUV and MLV samples are due primarily to differences in size and/or the number of lamella per liposome. Gruner et al. (1988) found that adding a few mole percent of lyso-PE and fatty acid, to simulate phospholipid degradation products, catalyzed the cubic phase formation for DOPE-Me. This is not an issue for our experiments, since the MLV and the LUV were treated identically. Significant lipid degradation is unlikely in our experiments, where the lipids are exposed to the elevated temperatures for less than 15 min. In the pH 9.5/20 mM Mg^{2+} buffer, it might be argued that the Mg^{2+} has less access to the interior of the MLV, relative to the LUV. However, in this case, the difference between the MLV and the LUV

samples is less than that observed at pH 4.5. Furthermore, at pH 4.5, all of the lipid in both the MLV and the LUV was rapidly exposed to protonation due to the rapid permeation of the acetic acid component of the buffer [ca. 2 s (Barbet et al., 1984; Bentz et al., 1987)].

Discussion

For several inverted phase forming lipids which show isotropic ^{31}P NMR resonances, the same set of intermembrane intermediates appear to produce both liposome fusion and a lipid phase transition.[2] We have shown this by correlating the temperature dependence of liposome fusion kinetics with that of the appearance of ^{31}P NMR isotropic resonances in the bulk dispersion of the lipid.

We believe that a plausible rationale can be constructed from the current knowledge of the phase behavior of these lipids and recent theoretical calculations about the dynamics of the transition from the L_α phase to the H_{II} phase. First, we will discuss the phase behavior of these inverted phase forming lipids and how our model of the phase transition explicates this behavior. Next, we will show how the fusion data and the theoretical model of the phase transition dynamics can be combined in a unified description.

Phase Behavior of the Lipid Systems. DSC experiments on the systems listed in Table I show a low-enthalpy transition consistent with an L_α/H_{II} phase transition in each system. The transition temperatures found for DOPE-Me at pH 4.5 or 7.4 agree well with the values reported by Gagné et al. (1985), Ellens et al. (1986b), and Gruner et al. (1988), and the value for DOPE/DOPC (3:1) at pH 4.5 (60 °C) agrees well with that of Tate and Gruner (1987) of 50–55 °C for a mixture slightly richer in PE (DOPE/DOPC = 10:3 mol/mol).

The X-ray diffraction patterns confirm that the calorimetrically observed transitions were to the H_{II} phase. The identity of the phases in (3:1) DOPE/DOPC is as found in (10:3) DOPE/DOPC (Tate & Gruner, 1987), and the lattice constants of the two phases agree well with the values in that report (59 Å in the L_α phase at 30 °C in both cases, 73 vs 71 Å in the H_{II} phase at 80 °C). There is no rigorous evidence in these diffraction patterns for the existence of other phases in these systems across the entire temperature range. However, we did find a slight broadening of the L_α phase diffraction peaks upon heating (most evident in the second-order peaks) which is consistent with the beginning of a transition to an inverted cubic phase (Gruner et al., 1988).

The appearance of narrow isotropic resonances in the ^{31}P NMR spectra of multilamellar lipid preparations is a very sensitive indicator for the presence of isotropic or inverted cubic phases.[4] Our own measurements and those of others indicate the presence of isotropic resonances in all these systems over temperature ranges beginning from 10–30 °C below T_H (see Table II).

Correlation of the Fusion Mechanism with the Phase Transition. As shown in Figure 3, the initial rates of fusion and contents leakage in the DOPE/DOPC (3:1) system at neutral pH and in the DOPE-Me system at pH 4.5 or in the presence of 20 mM Mg^{2+}/pH 9.5 all respond in the same way as the temperature is raised through ΔT_1 and T_H. The initial rates of fusion all increase dramatically as the temperature passes through the beginning of ΔT_1 (Table II), plateaus or goes through maxima at temperatures either just below or at T_H, and decreases thereafter. Initial leakage rates also start to increase within ΔT_1 but increase most dramatically (and surpass the rates of fusion) at temperatures $\geq T_H$.

These data agree with our previous results in that fusion sharply increases as the temperature reaches ΔT_1, for those systems which show isotropic resonances, and lysis sharply increases as the temperature reaches T_H (Bentz et al., 1985b; Ellens et al., 1986a,b). The observation that lipid mixing between the liposomes is rapid and extensive at temperatures above T_H is easily understood, as the membranes are being transformed to H_{II} phase structures. We do not call this process fusion, since there is no contents mixing involved.

Model of the $L_\alpha/I_{II}/H_{II}$ Phase Transition. Our data are consistent with a kinetic model of the L_α/H_{II} phase transition mechanism which is shown in Figure 8 (Siegel, 1986a–c; Siegel et al., 1988). Inverted micellar intermediates (IMI) formed between aggregated liposomes make the interfaces of the two membranes continuous, leading to lipid exchange in the outer monolayers. IMI formation is analogous to a subcritical fluctuation and should begin at temperatures well below T_H, particularly in systems with small L_α/H_{II} transition enthalpies like those described here. At T_H, IMI are proposed to aggregate in the plane of the two apposed membranes, forming other structures that result in rapid H_{II} phase formation and leakage (Siegel, 1986b).

In a particular subset of systems, where the ratio of the areas per lipid headgroup in the L_α and H_{II} phases is less than about 1.2 (Siegel, 1986b), or, equivalently, with "intermediate" values of the spontaneous radius of curvature described by Gruner et al. (1988), the IMI can form large numbers of another type of intermediate, denoted as an interlamellar attachment (ILA). ILA should yield isotropic ^{31}P NMR resonances, morphology like that of "lipidic particles" in freeze–fracture electron micrographs, and can assemble into inverted cubic phases (Siegel, 1986c). ILA formation should result in membrane fusion with mixing of aqueous contents. Hence, one expects to observe at least some fusion in unilamellar liposome dispersions under the same conditions in which bulk lipid–water samples exhibit isotropic ^{31}P NMR resonances. This is the case.

This model formally explains why only a subset of inverted phase forming lipids can form large numbers of form ILA, which we argue are responsible for the isotropic NMR resonances. It also predicts the formation of *some* ILA for *any* inverted phase forming lipid. Isotropic resonances are not always found for lipids which can achieve the H_{II} phase (Tilcock et al., 1984). However, Shyamsunder et al. (1988) have recently reported that pure DOPE, a lipid previously found to undergo a simple transition between the L_α and the H_{II} phase (Epand, 1985; Gagné et al., 1985), can be induced to form an inverted cubic phase by cycling the temperature above and below T_H several hundred times. Our model predicts that it is difficult for the ILA to revert to IMI (Siegel, 1986a), although the IMI can easily revert to apposed bilayers.

[4] The presence of <0.1-μm-diameter liposomes in the samples could also yield isotropic resonances. This is unlikely for two reasons. First, they would be fusing and would quickly reach sizes too large to produce isotropic motional averaging on the ^{31}P NMR time scale. Second, the samples were swelled from thin films of lipid, dispersed only by mild vortex mixing, and then pelleted. This should not produce any substantial number of small liposomes. We think that it is more likely that the isotropic resonances are produced by diffusion of the DOPE-Me molecules over the curved surfaces found in inverted cubic phases [e.g., see Siegel (1986c)]. Larsen et al. (1987) have shown that the motions of phospholipids with reorientational time constants of less than 20 μs produce isotropic ^{31}P NMR resonances in 109-MHz instruments. It can be shown that DOPE-Me diffusion on the surfaces of ILA (Figure 8) results in reorientational time constants of 4 μs or less, using a lipid diffusion coefficient for DOPE at 56 °C of 6×10^{-8} cm^2/s (Rilfors et al., 1986).

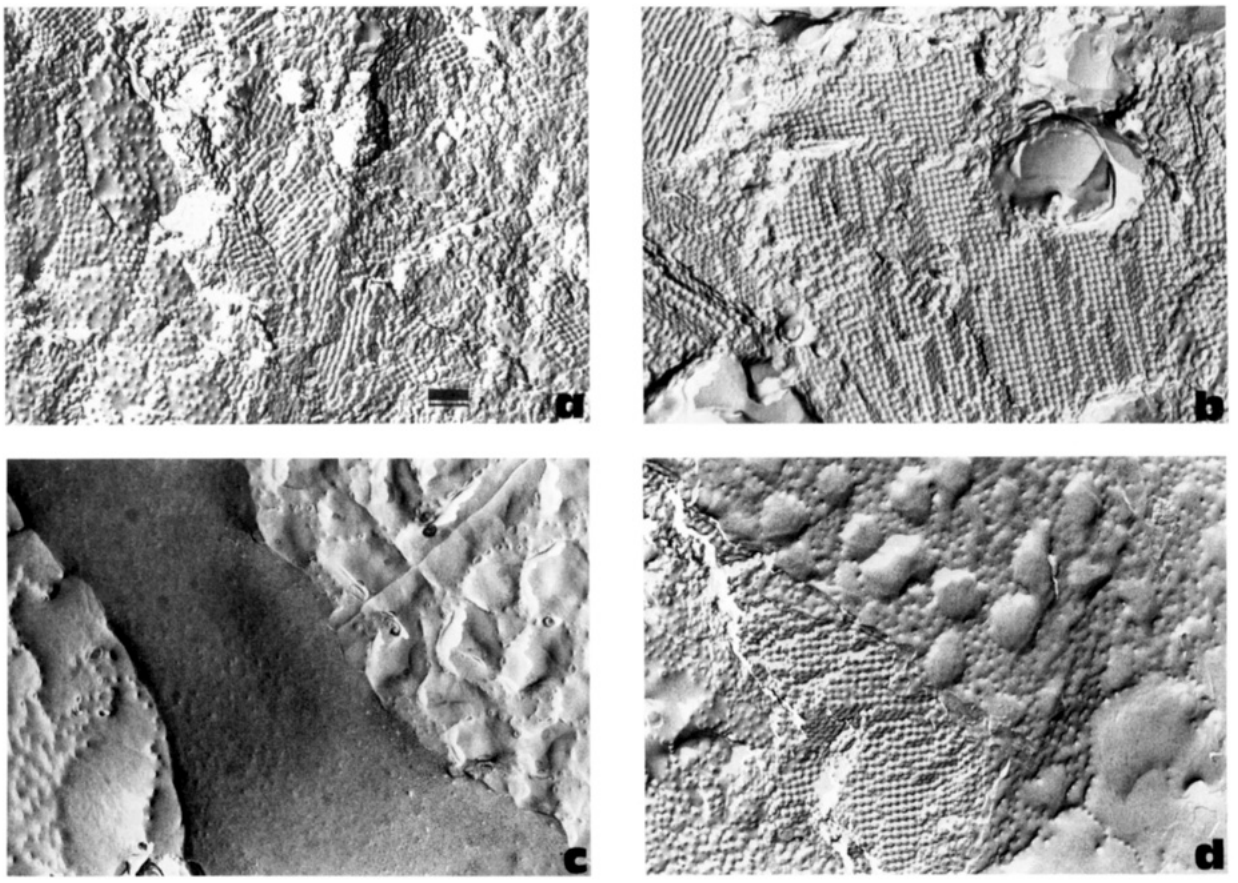

FIGURE 7: Freeze–fracture electron micrographs of DOPE-Me. Upper panels show the products of LUV (made by high-pressure extrusion at pH 9.5) obtained about 10 min after collapse due to lowering the pH to 4.5 at 55 °C (panel a) or adding 20 mM Mg/pH 9.5 at 45 °C (panel b). The lower panels show the corresponding cases when large MLV (diameter ~0.3–1.0 μm) are used initially. The MLV samples did not show collapse, but they were rapidly frozen after the same incubation time as the LUV samples. Thus, the MLV products in pH 4.5 at 55 °C (panel c) and with 20 mM Mg/pH 9.5 at 45 °C (panel d) are shown. In all cases, the lipid concentration was 200 μM. Therefore, the difference in the morphologies observed between the MLV and the collapse products of the LUV is due to the initial diameter and the number of lamella of the liposomes. All micrographs are at the same magnification. Bar: 150 nm.

Therefore, we would expect that each cycle through T_H would cause the accumulation of more ILA and the gradual formation of a cubic lattice, as has been found.

From the results of Shyamsunder et al. (1988), it is likely that the isotropic state/inverted cubic phase (I_{II}) can be induced in many, if not all, inverted phase forming lipids by the technique of exhaustively cycling the temperature through T_H. However, the correlation between the fusion mechanism and the isotropic state resides *only* in that subset of inverted phase forming lipids which show isotropic ^{31}P NMR resonances on the first heating scan. This is the situation which most closely corresponds to the initial contact between two liposomal membranes. The fact that pure DOPE requires hundreds of cycles through T_H to form many ILA, or whatever intermediates are responsible for the cubic lattices, simply proves that the initial interaction between two liposomes at $T \sim T_H$ is not likely to provoke the formation of enough ILA to produce measurable fusion.

In fact, not all of the fusion observed in PE-containing systems can be attributed to an ILA-mediated mechanism. Ellens et al. (1986a) showed that liposomes composed of DOPE, EPE, or TPE fused at temperatures below that of their respective values of T_H and that this fusion was abolished when the temperature exceeded T_H; i.e., only lysis and lipid mixing occurred. None of these lipids show isotropic resonances on the first heating scan. Furthermore, fusion could be observed as much as 35 °C below T_H, where no ILA and few, if any, IMI could form (Siegel, 1986b). The fusion observed 20 °C below ΔT_I for DOPE/DOPC (2:1) in Ellens et al. (1986a) cannot be ascribed to an ILA-mediated process. At ΔT_I, where the fusion rate for this system sharply increased, the ILA-mediated fusion mechanism became dominant. Thus, while we have elucidated one molecular mechanism for fusion here, we have shown also that it is not the only mechanism.

We reach the same conclusion with respect to leakage mechanisms. We have found that leakage also increases within ΔT_I, which implies a mechanism which depends upon the number of IMI or ILA. The model described by Figure 8 would predict leakage only via H_{II}-domain formation, which can only occur at temperatures at and above T_H. The leakage mechanism far below T_H is not known. It may be that the accumulation of a few defects (IMI or ILA) in the area of membrane apposition increases membrane permeability and/or susceptibility to rupture. It is known that leakage also can occur when adhesive forces promote excessive membrane tension and rupture, which has been demonstrated for very large (diameter >2 μm) liposomes (Kachar et al., 1986; Evans & Needham, 1986; Niles & Cohen, 1987). The leakage mechanism within ΔT_I may involve these forces, in addition to the defects formed by the IMI and ILA.

It has been proposed that lipidic particles have the same structure as we assign to IMI or ILA [see references in Verkleij (1984) and Cullis et al., (1985)]. Recently, ILA have been imaged directly using time-resolved cryotransmission electron microscopy (Talmon et al., 1989). Using the same technique, as well as freeze–fracture electron microscopy, Siegel et al.

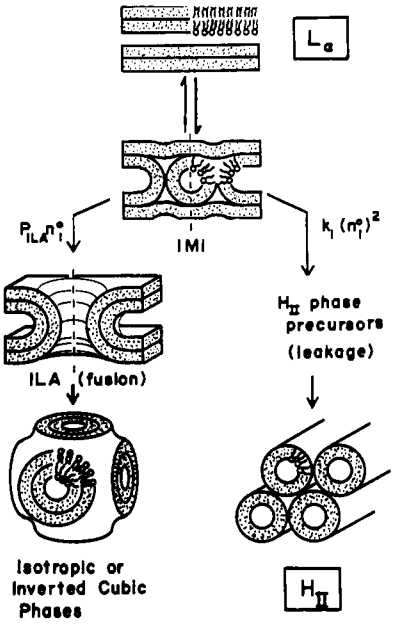

FIGURE 8: Proposed membrane–membrane interaction scheme. IMI are postulated *transient* intermediates in L_α/H_{II} phase transitions and can form at temperatures starting tens of degrees below T_H. H_{II} precursor formation is postulated to require aggregation of IMI between apposed bilayers at a rate $k_I n_I^{\circ 2}$, where k_I is a rate constant and n_I° is the steady-state surface density of IMI. The rate of ILA formation from single IMI is $P_{ILA}n_I^{\circ}$, where P_{ILA} is a rate constant determined by lipid structural parameters. As detailed in Siegel (1986a,c), n_I° and k_I increase and P_{ILA} decreases with temperature, so that H_{II} phase precursor formation proceeds faster than ILA formation as T approaches T_H. P_{ILA} is a very sensitive function of the spontaneous radius of curvature of the lipid system (Siegel, 1986b,c; Gruner et al., 1988). To a first approximation, P_{ILA} tends to be large in systems with large H_{II} tubes.

(1989c) showed that the lipidic particles in DOPE-Me (pH 7.4) are ILA and that its inverted cubic phase probably forms from ILA.

Collapse and Inverted Cubic Phase Formation. The collapse phenomenon is defined by three consistent observations. First, it occurs after aggregation and fusion. Second, it only occurs at temperatures within or slightly above ΔT_I. The incubation time before collapse decreases as the temperature approaches T_H, and at or above T_H, aggregation leads immediately to lysis. Third, the event is very hysteretic, in that the collapse time is variable, especially at temperatures far below T_H and at low lipid concentrations (Figure 6).

Figure 7 shows that the collapse product of the LUV suspension contains extensive domains of ordered lipidic particles. Previous studies have shown that inverted cubic phases form in systems exhibiting ordered arrays of lipidic particles (Boni & Hui, 1983; Hui et al., 1983; Lindblom et al., 1986; Rilfors et al., 1986). Figure 7 also shows that MLV incubated at low pH produce far fewer lipidic particles and *no* ordered arrays. With 20 mM Mg^{2+}/pH 9.5, the MLV do show some domains of aligned lipidic particles, indicating that the transition is more rapid with bound Mg^{2+}. Together, these data imply that the inverted cubic phase can be most quickly assembled by the accretion of small units of bilayer.

The size of the lattice observed in Figure 7 is interesting. The square lattice formed by the lipidic particles has a center-to-center distance of ca. 29 nm. Siegel (1986c) predicted that ILA assemble inverted cubic phases via the formation of an intermediate lattice of roughly the size and geometry observed in these micrographs. The inverted cubic phases of DOPE-Me typically have different symmetries and smaller lattice constants (Gruner et al., 1988). Presumably, at temperatures closer to T_H, the ILA lattice transforms into these inverted cubic phases. With respect to this, we have observed a small additional endotherm at ca. 60 °C in most, but not all, thermograms of DOPE-Me obtained at neutral or low pH, when the scan rate was 13 °C/h. This endotherm is larger in samples with greater lipid concentrations, and at slower scan rates (Siegel et al., 1989a). On the other hand, inverted cubic phase X-ray diffraction patterns are observed only after 20-h incubations at temperatures above 60 °C and below T_H (Siegel et al., 1989b).

Conclusion

The model in Figure 8 predicts that fusion occurs in the temperature interval ΔT_I, where the principal fate of the IMI is ILA formation. This is the temperature dependence for fusion which we observe. Thus, for these systems, the molecular mechanism for fusion is well understood. Essentially, fusion is an obligatory step in the transition to the cubic phase for these systems.

It is worth recalling that even the well-studied Ca^{2+}-induced fusion of phosphatidylserine (PS) liposomes has resisted a rigorous structural characterization (Bentz & Ellens, 1988; Bentz et al., 1988). We believe the difficulty in the structural analysis of the PS/Ca^{2+} fusion mechanism is not that intermembrane intermediates (of a different type than those proposed here) do not exist but rather that in the cases thus far studied, the lifetime of the intermediate is too short to permit resolution.

Inverted phase forming lipids are found commonly in biological membranes [see references in Siegel (1987) and Bentz and Ellens (1988)]. Bacteria will alter the proportion of inverted phase forming lipids in their membrane in response to environmental stress (Lindblom et al., 1986; Goldfine et al., 1987). Physiological levels of lipid metabolites (e.g., diacylglycerols) drastically alter the stability of inverted phases and the kinetics of liposome fusion (Siegel et al., 1989a). The passive roles of lipids in biological membranes (e.g., as a permeability barrier) are reasonably well understood. What roles, if any, these inverted phase forming lipids play in the dynamic function of biological membranes (e.g., vesicle fusion during exocytosis) have yet to be determined.

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Registry No. DOPE, 2462-63-7; DOPC, 10015-85-7; DOPE-Me, 87803-74-5; Mg, 7439-95-4.

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Physiological Levels of Diacylglycerols in Phospholipid Membranes Induce Membrane Fusion and Stabilize Inverted Phases†

David P. Siegel,*,‡ James Banschbach,‡ Dennis Alford,§ Harma Ellens,∥,⊥ Leonard J. Lis,# Peter J. Quinn,△ Philip L. Yeagle,◇ and Joe Bentz§

Procter and Gamble Company, Miami Valley Laboratories, Post Office Box 398707, Cincinnati, Ohio 45239-8707, Departments of Pharmacy and Pharmaceutical Chemistry and Department of Pharmacology, University of California, San Francisco, California 94143, Department of Physics and Liquid Crystal Institute, Kent State University, Kent, Ohio 44242, Department of Biochemistry, Kings College London, Campden Hill Road, Kensington, London W87AH, England, and Department of Biochemistry, State University of New York, Buffalo, New York 14214

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ABSTRACT: In the preceding paper (Ellens et al., 1989), it was shown that liposome fusion rates are substantially enhanced under the same conditions which induce isotropic ^{31}P NMR resonances in multilamellar dispersions of the same lipid. Both of these phenomena occur within the same temperature interval, ΔT_I, below the L_α/H_{II} phase transition temperature, T_H. T_H and ΔT_I can be extremely sensitive to the lipid composition. The present work shows that 2 mol % of diacylglycerols like those produced by the phosphatidylinositol cycle in vivo can lower T_H, ΔT_I, and the temperature for fast membrane fusion by 15-20 °C. N-Monomethylated dioleoylphosphatidylethanolamine is used as a model system. These results show that physiological levels of diacylglycerols can substantially increase the susceptibility of phospholipid membranes to fusion. This suggests that, in addition to their role in protein kinase C activation, diacylglycerols could play a more direct role in the fusion event during stimulus-exocytosis coupling in vivo.

Diacylglycerols are second messengers produced in cellular membranes by the PI cycle (Majerus et al., 1984, 1986; Sekar & Hokin, 1986) at levels of about 1 mol % with respect to phospholipid (Preiss et al., 1986). One known function of PI cycle diacylglycerols is to activate protein kinase C. It has been speculated that these diacylglycerols are also involved in exocytosis in that they make the relevant membranes more susceptible to fusion (Pickard & Hawthorne, 1978). We have shown that there is a liposome fusion mechanism associated with lamellar/inverted phase transitions (Ellens et al., 1989), and it is known that diacylglycerols lower lamellar/inverted phase transition temperatures (Dawson et al., 1984; Das & Rand, 1986; Epand, 1986; Coorsen & Rand, 1987). In this work, we studied the effect of 1-3 mol % diacylglycerols on the phase behavior and fusion kinetics of phospholipid systems that can form inverted phases.

Surprisingly, as little as 2 mol % diacylglycerols can lower the equilibrium lamellar/inverted hexagonal (L_α/H_{II})[1] phase transition temperature (T_H) of monomethylated dioleoylphosphatidylethanolamine (DOPE-Me)[1] by as much as 20 °C.

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‡ Procter and Gamble Company.
§ Departments of Pharmacy and Pharmaceutical Chemistry, University of California, San Francisco.
∥ Department of Pharmacology, University of California, San Francisco.
⊥ Present address: Drug Delivery Group, Smith, Kline & French Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939.
Kent State University.
△ Kings College London.
◇ State University of New York at Buffalo.

[1] Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; CHOL, cholesterol; DEG, dieicosenoin; DG, diacylglycerol; DLG, dilinolenin; DOG, diolein; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, monomethylated dioleoylphosphatidylethanolamine; DPA, dipicolinic acid; DPG, dipalmitin; DPX, *p*-xylylenebis(pyridinium bromide); DSC, differential scanning calorimetry; NTA, nitrilotriacetic acid; OAG, 1-oleoyl-2-acetylglycerol; OArG, 1-oleoyl-2-arachidonoylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; R_0, spontaneous radius of curvature of the monolayers of a lipid-water system, as defined by Gruner (1985); T_H, lamellar/inverted hexagonal (L_α/H_{II}) phase transition temperature; ΔT_I, range of temperatures at which isotropic ^{31}P NMR resonances are observed from fully hydrated bulk multilamellar preparations of a lipid system; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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