Fluorometric detection of the bilayer-to-hexagonal phase transition in liposomes

ARTICLE *in* BIOCHEMISTRY · JUNE 1988

Impact Factor: 3.02 · DOI: 10.1021/bi00411a009 · Source: PubMed

4 AUTHORS, INCLUDING:

Theresa M Allen
University of British Columbia - Vancouver
258 PUBLICATIONS **18,189** CITATIONS

SEE PROFILE

Fluorometric detection of the bilayer-to-hexagonal phase transition in liposomes

Keelung Hong, Patricia A. Baldwin, Theresa M. Allen, and Demetrios Papahadjopoulos

Biochemistry, **1988**, 27 (11), 3947-3955 • DOI: 10.1021/bi00411a009

Fluorometric Detection of the Bilayer-to-Hexagonal Phase Transition in Liposomes†

Keelung Hong,*,‡ Patricia A. Baldwin,‡,§ Theresa M. Allen,‡,∥ and Demetrios Papahadjopoulos‡,⊥

Cancer Research Institute and Department of Pharmacology, University of California, San Francisco, San Francisco, California 94143

Received August 19, 1987; Revised Manuscript Received January 22, 1988

ABSTRACT: We have used the fluorescent probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) to detect the bilayer-to-hexagonal phase transition. The fluorescence intensity of the probe was found to increase during the bilayer-to-hexagonal transition. The bilayer-to-hexagonal transitions of various types of phosphatidylethanolamine or cardiolipin measured by this method are consistent with results obtained by differential scanning calorimetry. To establish this method for wider use, agents known to alter the bilayer-to-hexagonal transition were examined, and the results are comparable with the published data. The added advantage of this fluorometric method over other currently available techniques is that it is applicable not only for aggregated lipid samples but also for dilute liposome suspensions. This is especially important when one of the components of the system under study can partition between lipid and aqueous phase. Since NBD is located near the headgroup region of the bilayer, it most likely detects the change of the environment surrounding that region. On the basis of our present study, it appears that NBD-PE is sufficiently sensitive to detect bilayer-to-hexagonal phase transition.

In recent years, there has been renewed interest in lipids which are capable of undergoing a transition from bilayer to nonlamellar phases. Nonlamellar structures such as the inverted hexagonal (H_{II}) phase are found under certain conditions when isolated lipids are dispersed in water (Luzzati, 1968; Cullis & De Kruijff, 1979; Verkleij, 1984). This polymorphic phase change is a function of temperature, hydration, and the ionic concentration of the aqueous medium. Although Verkleij (1984) and others have suggested that membrane processes such as membrane fusion, synthesis, and transport include a nonbilayer phase, direct proof of this involvement has not yet been presented. The polymorphic phase behavior of a lipid or lipid mixture is generally detected by ^{31}P NMR, X-ray diffraction, differential scanning calorimetry (DSC),[1] freeze–fracture electron microscopy, or, more recently, infrared spectroscopy (Cullis & De Kruijff, 1979; Rand & Sengupta, 1972; Harlos & Eibl, 1981; Seddon et al., 1983; Casal & Mantsch, 1984). However, using these techniques, it is virtually impossible to determine bilayer to nonlamellar phase transitions in dilute membrane suspensions or to analyze the kinetics of a bilayer to nonlamellar phase transition. In this study, we present a method using the fluorescent probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) which can detect the bilayer-to-hexagonal (H_{II}) phase transition.

Changes in the fluorescence intensity and/or polarization of fluorophores have long been used to determine the gel to liquid-crystalline transition in membrane systems (Sklar et al., 1977; Faucon & Lussan, 1973; Shinitzky & Barenholz, 1978). In the work presented here, we have investigated the change in fluorescence intensity of NBD-PE incorporated in liposomes using several lipid systems known to undergo bilayer-to-hexagonal phase transitions. An increase of NBD-PE fluorescence was found to correlate with the transition of bilayer-to-hexagonal phase. Additionally, we have used this new method to study the effects of two compounds known to promote or inhibit the formation of the hexagonal phase. In the present investigation, we demonstrate two ways to apply the fluorometric technique to the study of thermal behavior of lipids. For a dilute liposome suspension, the fluorescence intensity is monitored at constant temperature over a period of time after the addition of ions which are known to induce a lipid phase transition. A time-dependent increase of the fluorescence intensity after ion addition is an indication of the hexagonal phase formation. For a preaggregated lipid sample, a continuous scan of fluorescence monitored in a front face mode vs temperature is performed. The bilayer-to-hexagonal transition is the temperature region where the fluorescence intensity rises in a heating scan. The transition temperatures of several lipids determined by these fluorometric approaches correlate well not only with our data from DSC but also with published data using other methods.

Materials and Methods

Lipids. All lipids used in this study were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). The lipids were

† This investigation was supported by NIH Grant GM-28117, by American Cancer Society Postdoctoral Fellowship PF-2331 (P.A.B.), by MRC Grant MA-9127, by the Alberta Heritage Foundation for Medical Research (T.M.A.), and in part by American Heart Association Grant-in-Aid 83-636 and a grant from the Academic Senate of the University of California, San Francisco.

* Address correspondence to this author at the Cancer Research Institute, M-1282, University of California, San Francisco.

‡ Cancer Research Institute.

§ Present address: California Biotechnology, Inc., 2450 Bayshore Parkway, Mountain View, CA 94043.

∥ Permanent address: Pharmacology Department, University of Alberta, Edmonton T6G 2H7, Canada.

⊥ Department of Pharmacology.

[1] Abbreviations: BHT, butylated hydroxytoluene; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; MLV, multilamellar vesicle(s); NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SUV, small unilamellar vesicles; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; TPE, phosphatidylethanolamine, transesterified from egg phosphatidylcholine; T_H, bilayer-to-hexagonal transition temperature; EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-3947$01.50/0

stored as chloroform solutions in sealed ampules under argon at −70 °C.

Chemicals. Sodium deoxycholate was purchased from Sigma (St. Louis, MO). Butylated hydroxytoluene (BHT), 99+% pure, was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Preparation of Liposomes. Multilamellar vesicles (MLV) containing 0.1–1.0 mol % NBD-PE were prepared by mixing the lipid and probe in solvent, followed by solvent evaporation under vacuum. After addition of a small volume of buffer to the dried lipid, the sample was vortexed for 5 min. Small unilamellar vesicles (SUV) used in some experiments were prepared by sonication. Lipid suspensions were sonicated for 15 min in a bath sonicator at room temperature. Sonication was done in sealed tubes under argon [see Bangham et al. (1974) for detailed SUV preparation]. The liposome concentration was determined by measuring lipid phosphorus according to the published procedure of Bartlett (1959). The buffers used were 5 mM borate/150 mM NaCl/0.1 mM EDTA, pH 9.5 (pH 9.5 buffer), 5 mM TES/150 mM NaCl/0.1 mM EDTA, pH 7.4 (pH 7.4 buffer), and 10 mM citrate/150 mM NaCl/0.1 mM EDTA, pH 5.0 (pH 5.0 buffer). Ca^{2+} was added to the buffer from a 1 M $CaCl_2$ solution. A concentrated (1 M) citric acid solution (pH 3.3) was used to lower the pH as desired. BHT in ethanol (10 mM) was added to the chloroform solution of lipids prior to the solvent evaporation. Liposomes containing BHT were made by vortexing the dry BHT–lipid mixture with buffer. A concentrated deoxycholate solution (0.24 M) was added to the dry lipid mixture together with the buffer before vortexing to form multilamellar vesicles.

Fluorescence Measurements. Fluorescence intensity was monitored by using a SPEX Fluorolog 2 either in right (90°) detection angle or in front face mode (22.5° detection angle from the excitation beam). The excitation wavelength was 455 nm (1.8-nm slit), and the emission wavelength was 530 nm (2.25-nm slit) unless otherwise specified. The temperature of the sample was controlled with a circulating water bath (heating rate approximately 1–3 °C/min; cooling rate 1 °C/min) and was monitored by a copper–constantan thermocouple in the cuvette. Both fluorescence intensity and temperature during heating or cooling scans were continuously recorded on a Bascom–Turner electronic storage recorder (Model 3120). For liposome suspensions which had not been preaggregated with low pH or Ca^{2+}, the measurement of fluorescence was performed in a regular quartz cuvette (1-cm path length) using the right angle mode. The fluorescence measurement of aggregated samples was taken by using the front face mode. The aggregated lipid was first spun down in an Eppendorf centrifuge, and the pellet was evenly placed in a short path-length (0.1 mm) quartz cuvette. For each measurement in the front face mode, 5–10 μmol of phospholipid was used. For reasons of clarity, *relative fluorescence intensity* was used in most of the scan tracings. The traces of the fluorescence intensity vs temperature were offset along perpendicular axes to avoid crossover or overlapping.

Vesicle Aggregation. The fluorometer is equipped with two 90° emission channels, allowing fluorescence and light scattering to be measured simultaneously when it is desired. The aggregation of liposomes was monitored by light scattering at 455 nm in the second channel using a 450-nm band-pass filter (448–454-nm 50% transmission) to avoid any fluorescence coming to this channel.

Differential Scanning Calorimetry. Thermograms were taken on a Perkin-Elmer DSC-2 using a scan speed of 5 °C/min. The transition temperature in the DSC thermograms was taken as the intercept of the tangent of the rising slope with the base line.

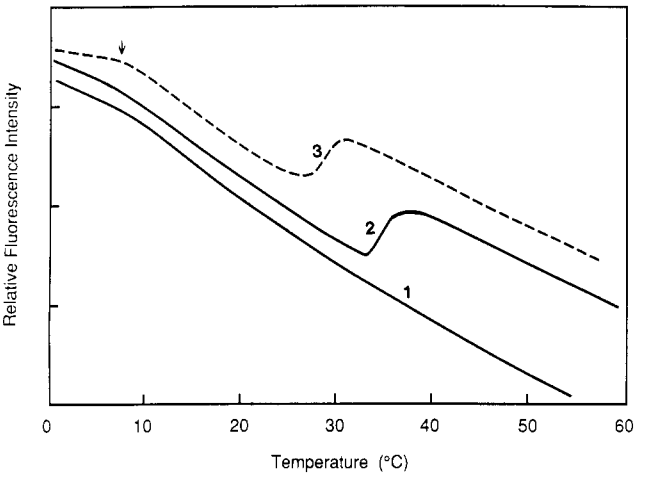

FIGURE 1: Continuous scans of the fluorescence intensity of NBD-PE-labeled egg PE as a function of temperature. Multilamellar vesicles of egg PE containing 0.1% NBD-PE were prepared as described under Materials and Methods. (1) Heating scan of 100 μM egg PE MLV suspension using the right angle mode of detection at pH 9.5. A discontinuity representing the transition of the gel-to-liquid-crystalline phase was around 9–10 °C. (2) Heating scan of aggregated egg PE MLV at pH 5.0 (solid line); T_H = 34–37 °C. (3) Subsequent cooling scan of sample 2 (dashed line); T_H = 26–30 °C. The transition temperature of the gel-to-liquid-crystalline phase is indicated by an arrow (6–7 °C).

RESULTS

Continuous Scans of Fluorescence Intensity vs Temperature. The fluorescence intensity of a fluorophore in solution is generally a continuous negative function of temperature. However, for a fluorophore incorporated into a membrane, discontinuities in this pattern of decline with temperature are found to correspond to transitions between one lipid phase and another (Sklar et al., 1977; Faucon & Lussan, 1973; Shinitzky & Barenholz, 1978). Figure 1 shows the scans of NBD-PE fluorescence intensity vs temperature on MLV of egg PE (100 μM). At pH 9.5, this lipid is not aggregated and does not undergo a transition to the hexagonal phase (curve 1 in Figure 1). However, after dialysis against pH 5 buffer for 5 h at room temperature under argon, the resulting aggregated egg PE showed a rising break in the curve of the heating scan (curve 2 in Figure 1). The change in slope at approximately 10 °C seems to be associated with the main (gel-to-liquid-crystalline) bilayer transition (see Figure 2). The rising break between 33 and 38 °C correlates well with the bilayer-to-hexagonal phase transition determined by DSC on similarly prepared samples. A subsequent cooling scan was routinely performed after a heating scan. In general, both curves have similar characteristics, except that the bilayer-to-hexagonal transition temperature seen in the cooling scan using this method was lower than that of the heating scan. For egg PE at pH 5, the transition in the cooling scan occurs between 26 and 30 °C (curve 3 in Figure 1).

To further characterize the changes in NBD-PE fluorescence during phase transitions, a series of control experiments were performed on DMPC, a lipid which does not undergo the bilayer-to-hexagonal transition. There was no detectable difference in emission spectra at low pH, indicating that H^+ did not interact directly with the probe molecule. The scans of fluorescence vs temperature of DMPC (MLV) containing NBD-PE at different pHs are shown in Figure 2. Two points of discontinuity were observed in a continuous scan of the

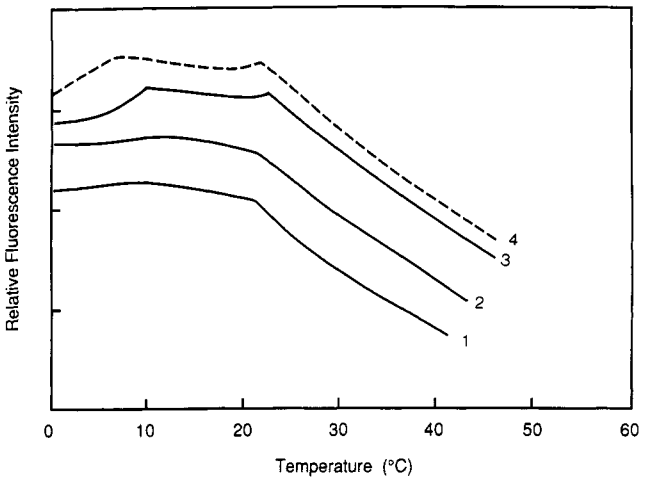

FIGURE 2: Continuous scans of the fluorescence intensity of DMPC containing NBD-PE as a function of temperature. DMPC MLV were prepared in a buffer as described under Materials and Methods, and the fluorescence intensity was monitored with the right angle mode. The concentration of lipid in each sample was 100 μM. (1) Heating scan of DMPC (0.1% NBD-PE) in the presence of 10 mM Ca^{2+} at pH 9.5. (2) Heating scan of DMPC (0.1% NBD-PE) at pH 5.0. (3) Heating scan of DMPC (1% NBD-PE) at pH 9.5. (4) Subsequent cooling scan of sample 3.

fluorescence intensity of DMPC containing 1 mol % NBD-PE (traces 3 and 4). These two points of discontinuity separated the scan trace into three segments of different slopes. These two breaking points correspond respectively to the pretransition and main transition of the gel-to-liquid-crystalline phase measured by DSC. The conformational alterations of the headgroup region at the pretransition and the main transition of DMPC appear to affect the NBD fluorescence. It should be noted that the response of NBD-PE to the main bilayer transition (gel-to-liquid-crystalline transition) was different from that due to the bilayer-to-hexagonal phase transition (compare Figures 1 and 2). Above the main transition temperature of DMPC, the plot of NBD-PE fluorescence versus temperature *decreases* with a much steeper slope than is observed below the main transition temperature. A similar profile also appeared on the scan tracing of egg PE; the discontinuity representing the main transition was seen at the lower temperature region (Figure 1). In contrast, the NBD-PE fluorescence *increases* with increasing temperature during the transition from the bilayer-to-hexagonal phase. A decline of the fluorescence intensity of NBD-PE in DMPC as the temperature was lowered below the main transition temperature was not expected. A possible explanation is that NBD-PE is partially phase-separated in this low-temperature region and the local concentration of NBD-PE is high enough to be slightly self-quenched. This was also seen below the main phase transition of egg PE containing 1 mol % NBD-PE. In subsequent experiments, we found that this self-quenching phenomenon was greatly diminished by lowering the probe concentration to 0.1 mol % (curves 1 and 2 of Figure 2).

Addition of Ca^{2+} is known to induce formation of the hexagonal phase in egg PE even at relatively high pH (Ellens et al., 1986). To compare the temperature of the Ca^{2+}-induced hexagonal transition with that induced by low pH, we carried out experiments on the same preparation of egg PE MLV containing 1 mol % NBD-PE. Ca^{2+}-induced hexagonal transition of egg PE MLV at pH 9.5 was observed in the same temperature range as that at pH 5.0. To demonstrate that the observed fluorescence changes are not due to a direct interaction of Ca^{2+} with NBD-PE, we incorporated 1 mol % NBD-PE into egg PC MLV. As expected, the scans of

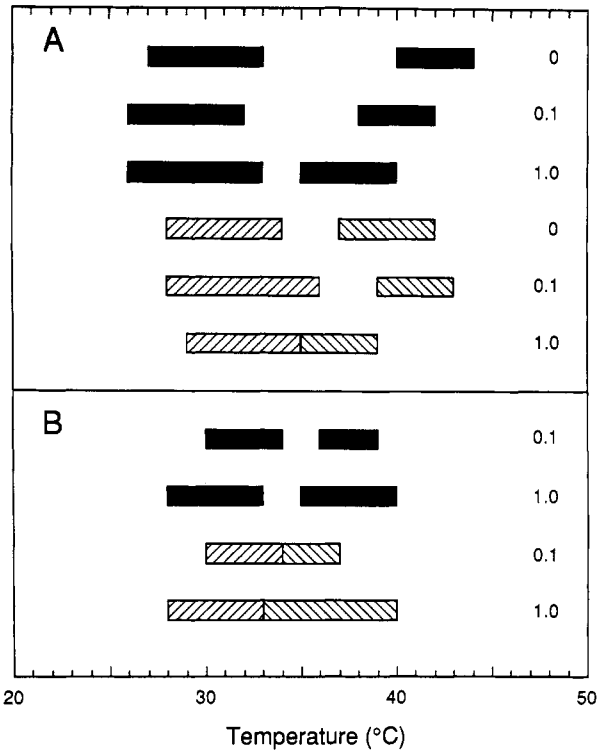

FIGURE 3: Comparison of the bilayer-to-hexagonal transitions of the same batch of egg PE measured by DSC (A) or the fluorometric method (B). Egg PE MLV containing 0, 0.1, or 1 mol % NBD-PE was prepared in pH 9.5 buffer as described under Materials and Methods. Each sample containing the same amount of NBD-PE was then divided into two (0% NBD-PE) or four (0.1 and 1 mol % NBD-PE) for either Ca^{2+} or low-pH treatment. Solid bars represent T_H of egg PE at pH 5.0. Hatched bars represent T_H of egg PE treated with 20 mM Ca^{2+} at pH 9.5. Heating scans are shown on the right, cooling scans on the left.

fluorescence intensity vs temperature of egg PC MLV in the presence of 10 mM Ca^{2+} did not show breaks similar to that of the hexagonal transition in egg PE MLV. An additional control on DMPC at pH 9.5 in the presence of 10 mM Ca^{2+} was performed, and the result shows two break points, indicating the pretransition and main transition, respectively, as was observed at low pH (curve 1 of Figure 2).

In order to establish this fluorometric method for measuring bilayer-to-hexagonal phase transition, the effect of the probe molecules on the transition temperature was examined. Since the bilayer-to-hexagonal phase transition of egg PE varies among different lots of PE preparations, a direct comparison between DSC and the fluorometric method was performed on the *very* same preparation of liposomes. Figure 3 shows the results of these experiments. By including NBD-PE in egg PE MLV, for the DSC scans, the effect of NBD-PE on the bilayer-to-hexagonal transition can be directly measured. The transition temperatures of egg PE measured by DSC are shown in Figure 3A. It appears that NBD-PE does not broaden the transitions. However, a clear trend was observed that NBD-PE lowered the transition induced by low pH *in the heating scans*. The temperature of Ca^{2+}-induced phase change was also lowered by the presence of 1% NBD-PE *in the heating scan*. Lower concentration of NBD-PE (0.1%) has almost no effect on the Ca^{2+}-induced transition. It is noteworthy that the effect of NBD-PE we observed in the heating scans was not seen in the cooling scans. Thus, the presence of NBD-PE mainly reduces the hysteresis (the difference in transition temperatures measured between heating and cooling scans) of egg PE; i.e., NBD-PE as an impurity, breaks the metastable state of the superheated bilayer state. In contrast to DSC results, fluo-

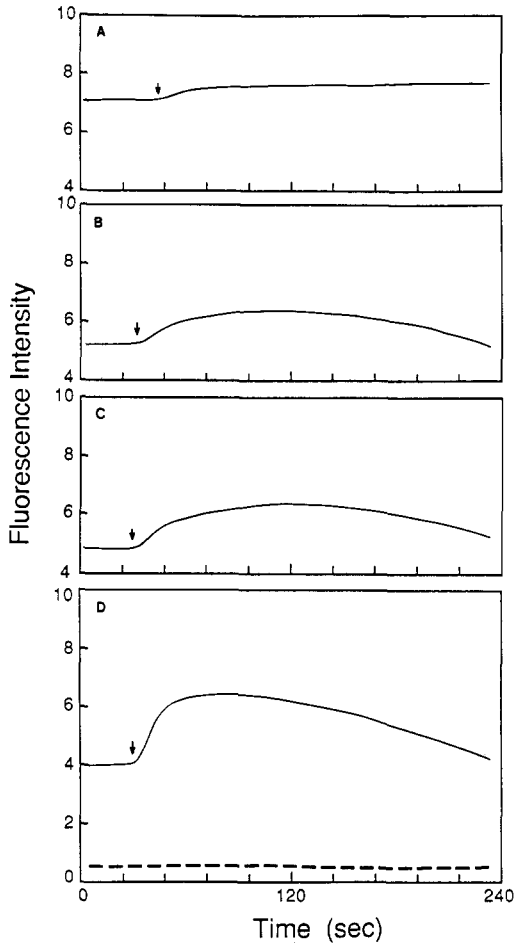

FIGURE 4: Time course of the changes of NBD-PE fluorescence intensity in egg PE in response to added protons as a function of temperature. Egg PE MLV containing 0.1 mol% NBD-PE were prepared in borate buffer (pH 9.5) as described under Materials and Methods. For each curve, a 100 μM egg PE sample was equilibrated with stirring in a cuvette at the indicated temperature. Concentrated citric acid (pH 3.3) was injected into the cuvette at the time indicated by the arrow to lower the pH of the sample. Right angle fluorescence was observed as a function of time (excitation, 455 nm, 1.8-nm slit; emission, 530 nm, 2.55-nm slit). A series of parallel experiments were performed in nonlabeled egg PE MLV to determine the contribution of light scattering to the fluorescence channel (dashed line). The temperatures at which the fluorescence intensity was monitored were (A) 10.7 °C, (B) 29 °C, (C) 34 °C, and (D) 40 °C. The y axis represents the actual fluorescence intensity in 10^3 photon counts/s.

rometric data show a smaller hysteresis in both H^+- and Ca^{2+}-induced phase change. Despite a slight difficulty in comparing the transition temperatures obtained by one method versus the other due to the broadness of the bilayer-to-hexagonal transition, a consistent trend exists that the transition temperatures measured by fluorometric method tend to be a few degrees lower than those measured by DSC.

Bilayer-to-Hexagonal Phase Transition Induced by Ions in a Dilute Vesicle Suspension. In order to use this fluorometric method to study the bilayer-to-hexagonal phase transition in a dilute vesicle suspension, we monitored the change over time in fluorescence intensity of NBD-PE incorporated in MLV composed of egg PE in response to a decrease in pH at a series of temperatures. A typical temperature-dependent response of the fluorescence intensity to a change in pH from 9.5 to 5.0 is shown in Figure 4A–D. In a separate series of experiments, 90° light scattering was measured along with the fluorescence intensity to observe the extent of liposome aggregation. An increase in light scattering was observed, indicating that vesicle aggregation occurred at all temperatures.

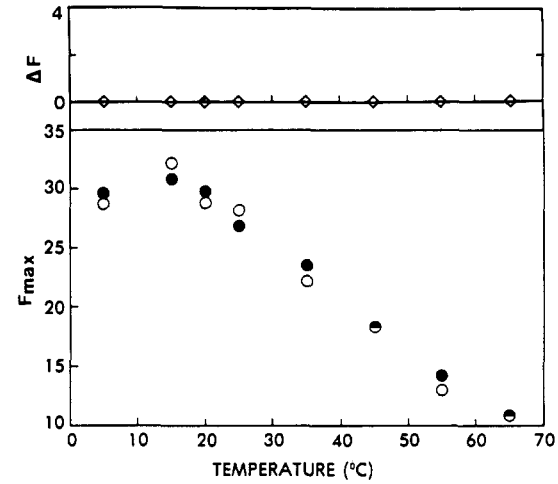

FIGURE 5: Fluorescence intensity of dilute suspensions of NBD-PE-labeled (1%) MLV of DMPC before and after ion addition as a function of temperature. DMPC MLV were prepared as described under Materials and Methods. At each temperature, a curve like those shown in Figure 4 was obtained by using an injection of $CaCl_2$ to a final concentration of 10 mM or an injection of a concentrated citric acid solution (pH 3.3) to lower the pH to 5. (Lower panel) The plotted points represent the actual fluorescence intensity measured in 10^4 photon counts/s. (●) DMPC MLV before and after Ca^{2+} addition; (○) DMPC MLV before and after lowering the pH to 5. (Upper panel) Using the data from the lower panel, we plotted the change in fluorescence intensity resulting from ion addition at each temperature. Since there was no change of the fluorescence intensity after ion injection, ΔF was zero for all the measurements.

However, a large increase in fluorescence was observed only at or above 34 °C upon the addition of H^+ to NBD-PE-labeled egg PE MLV. Since the vesicles aggregated after the addition of H^+, there was a possibility that an increase in light scattering might affect the fluorescence measurement. A series of parallel experiments using nonlabeled egg PE MLV was carried out under the same experimental conditions. A typical result is shown in Figure 4D; there was no detectable change in the fluorescence channel due to the increase of light scattering.

A similar series of experiments was undertaken to examine the response of egg PE to Ca^{2+} over a temerature range when probed with 0.1 mol % NBD-PD (data not shown). A characteristic rise in fluorescence intensity was observed above 43 °C after the addition of 10 mM Ca^{2+} to 100 μM liposomes of egg PE. As a control, experiments were performed with DMPC containing 0.1% or 1% NBD-PE at pH 9.5 before and after the addition of Ca^{2+}, as well as before and after lowering the pH to 5. No change of fluorescence intensity was observed after ion addition at all temperatures. This again assured us that there was no direct interaction of Ca^{2+} or H^+ to NBD-PE that resulted in changes in the fluorescence intensity.

We have designated F_0 as the actual fluorescence intensity observed *before* ion addition at each temperature and F_{max} as the maximum fluorescence intensity reached *after* ion addition. In principle, F_{max} should represent the fluorescence of the equilibrium state under the new ionic conditions. For each vesicle suspension at each temperature, F_0 and F_{max} were measured and plotted as a function of temperature. As expected for DMPC containing 1 mol % NBD-PE at pH 9.5 above the gel-to-liquid-crystalline transition, before and after the addition of Ca^{2+}, and before and after lowering the pH to 5, a smooth decline of the fluorescence intensity with increasing temperature was observed under all ionic conditions (Figure 5). In contrast, the results for NBD-PE in egg PE before and after injection of H^+ (Figure 6A) or Ca^{2+} Figure 6B) showed a break in the decline of the fluorescence intensity

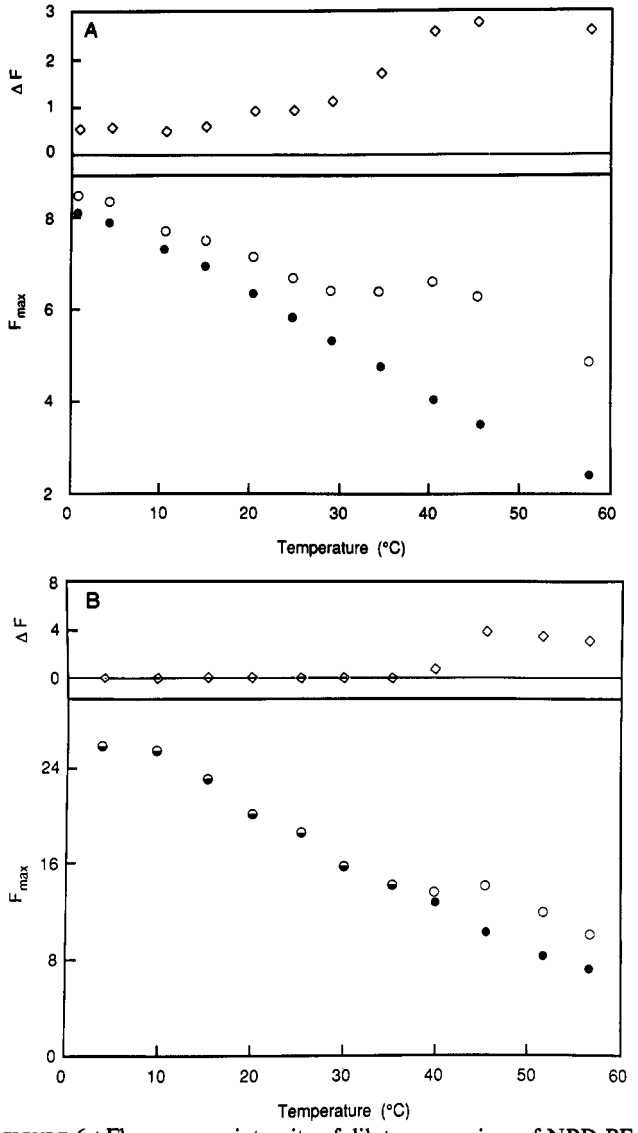

FIGURE 6: Fluorescence intensity of dilute suspensions of NBD-PE-labeled egg PE vesicles before and after ion addition as a function of temperature. At each temperature, a curve like those shown in Figure 4 was obtained using an injection of $CaCl_2$ to a final concentration of 10 mM at pH 9.5 or an injection of a concentrated citric acid solution (pH 3.3) to lower the pH from 9.5 to 5.0. (Lower panels) The plotted points represent F_{max} (○) and F_o (●) in 10^3 photon counts/s. F_o is the fluorescence intensity before ion addition, and F_{max} is the maximum fluorescence intensity observed after ion addition. (Upper panels) Using the data from the lower panels, we plotted the *change* in fluorescence intensity (ΔF) resulting from ion addition at each temperature. (A) Egg PE MLV (0.1 mol % NBD-PE) before and after H^+ addition; i.e., the pH of the sample was lowered from 9.5 to 5.0. (B) Egg PE MLV (1 mol % NBD-PE) before and after Ca^{2+} addition; the pH of the sample was maintained at 9.5.

vs temperature curve. The increase of NBD-PE fluorescence in egg PE in response to ion addition correlates very well with that in the continuous heating scan of samples which had been preaggregated by dialysis against protons or calcium (see Figures 1 and 3). The change of fluorescence intensity, ΔF, was the difference between F_{max} and F_o, i.e., $\Delta F = F_{max} - F_o$. For DMPC containing NBD-PE, ΔF's were zero, and a straight line was obtained by plotting ΔF vs temperature (upper panel of Figure 5). A sigmoidal curve was obtained when ΔF of egg PE liposomes was plotted as described. Low-pH- and Ca^{2+}-induced phase changes of dilute suspensions of egg PE liposomes are shown in the upper panels of Figure 6. The transition temperature was taken as the range over which the fluorescence intensity changed from one region of constant slope to a second region of constant slope. To verify this method on PE's with different acyl chain composition, similar experiments were performed on *Escherichia coli* PE, and on PE which has been prepared by transesterification of egg phoshatidylcholine (TPE). These results are shown in Table I along with corresponding DSC data. It should be noted that the physical states of the samples were not comparable; i.e., preaggregated PE was used for DSC, and dilute vesicle suspensions were used for fluorometric measurements. As shown in Table I, transitions obtained by DSC appear to be higher than those by fluorometric measurements. Whether this difference can be attributable mainly to the inherent difference in methodology or including the sample's physical state remains to be determined.

Table I: Bilayer-to-Hexagonal Phase Transition Temperatures (T_H) As Determined by NBD-PE Fluorescence and Calorimetry[a]

lipid	ionic conditions	T_H by fluorescence (°C)	T_H by DSC (°C)
DMPC	10 mM $CaCl_2$, pH 9.5	not obsd	not obsd
DMPC	pH 5	not obsd	not obsd
egg PE	10 mM $CaCl_2$, pH 9.5	38–43	44
egg PE	pH 5	30–40[b]	41
TPE	10 mM $CaCl_2$, pH 9.5	53–65	69
TPE	pH 5	53–64	67
E. coli PE	10 mM $CaCl_2$, pH 9.5	54–68	68
E. coli PE	pH 5	50–64	66

[a] The transition temperatures obtained by using the fluorometric method were determined in experiments like those shown in Figure 4. The transition temperature was taken as the range over which the fluorescence intensity changed from one region of constant slope to a second region of constant slope. Since data points were taken only every 5 °C, there is an error of ±3 °C in the temperatures reported as the onset and end points of the transition range. Samples for DSC (which included 1 mol % NBD-PE) were prepared as described under Materials and Methods, except that a larger quantity of lipid was used. Typically, each sample contained 100 μM lipid for fluorometry and 8 mg of lipid for DSC. Thermograms were taken on a Perkin-Elmer DSC-2 using a scan speed of 5 °C/min. The transition temperature in the DSC thermograms was taken as the intercept of the tangent of the rising slope with the base line. [b] Egg PE used in this measurement is a different batch from that which was used for the measurement shown in Figure 1; therefore, T_H is different from that measured in Figure 1.

Agents Affecting Lipid Polymorphic Phase Behavior. To establish a wider use of this fluorometric method of detection of the bilayer-to-hexagonal transition, we examined two agents, BHT and deoxycholate, known to affect the lipid polymorphic phase behavior (Chen et al., 1986; Madden & Cullis, 1982). The results are shown in Figure 7. NBD-PE-labeled (0.1 mol %) egg PE vesicles containing 5 mol % deoxycholate at pH 5.0 had a sharp bilayer-to-hexagonal transition at 38–42 °C (curve 1), which was significantly higher than the control 33–38 °C seen for pure egg PE (curve 2 in Figure 7). A higher transition temperature was also consistently seen in the cooling scan, 32.5–37.5 vs 26–30 °C (curve 3 in Figure 7 compared to curve 3 in Figure 1). Deoxycholate did not broaden the transition, but the elevation of the transition of egg PE by deoxycholate was found to depend on the amount of deoxycholate added. This result is consistent with earlier findings obtained by ^{31}P NMR (Madden & Cullis, 1982).

A heating scan of NBD-PE-labeled egg PE containing 10 mol % BHT at pH 5.0 showed a slow rise of fluorescence intensity. The bilayer-to-hexagonal transition was very broad, ranging from 12 to 27 °C (curve 4 in Figure 7). However, the cooling scan (curve 5 in Figure 7) showed a sharp break at 13–17 °C. Both heating and cooling scans have transition temperatures much lower than egg PE without added BHT. A similar effect of BHT on the bilayer-to-hexagonal transition was observed earlier by different methods (Cheng et al., 1986).

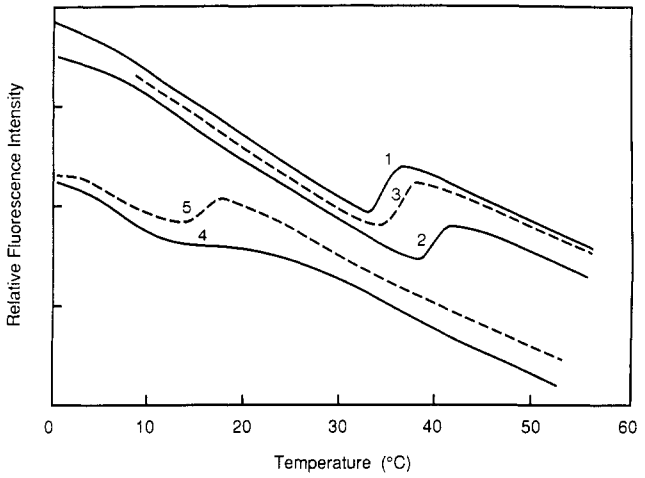

FIGURE 7: Continuous scans of the fluorescence intensity of NBD-PE-labeled egg PE as a function of temperature in the presence of BHT or deoxycholate. Egg PE MLV containing 0.1 % NBD-PE were prepared in the presence of either BHT or deoxycholate in pH 9.5 buffer. A concentrated citric acid solution (pH 3.3) was injected to the vesicle suspension to lower the pH to 5.0. The aggregated lipids were centrifuged, and the pellet was used in a front face fluorescence scan. (1) Heating scan of egg PE at pH 5.0 (solid line); T_H = 33–38 °C. (2) Heating scan of egg PE containing 5 mol % deoxycholate at pH 5.0 (solid line); T_H = 38–42 °C. (3) Subsequent cooling scan of sample 2 (dashed line); T_H = 32.5–37.5 °C. (4) Heating scan of aggregated egg PE MLV in the presence of 10 mol % BHT at pH 5.0 (solid line); T_H = 12–27 °C. (5) Subsequent cooling scan of sample 4 (dashed line); T_H = 13–17 °C.

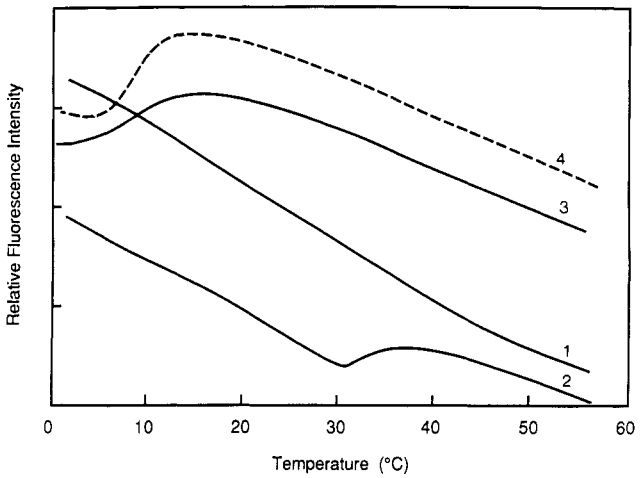

FIGURE 8: Continuous scan of the fluorescence intensity of NBD-PE-labeled SUV of PC–cardiolipin (1:1) as a function of temperature in the presence of Ca^{2+}. Small unilamellar vesicles containing 0.2 mol % NBD-PE were prepared in pH 7.4 buffer as described under Materials and Methods. Ca^{2+} solution was added to the vesicle suspension with a mole ratio of Ca^{2+} to lipid of 1:1. The aggregated Ca^{2+}–lipid complex was scanned in the fluorometer with front face mode. (1) Heating scan of *E. coli* cardiolipin–PC (1:1) liposomes in the absence of Ca^{2+}. (2) Heating scan of *E. coli* cardiolipin–PC (1:1) liposomes in the presence of 10 mM Ca^{2+}; T_H = 30–45 °C. (3) Heating scan of bovine heart cardiolipin–PC (1:1) liposomes in the presence of 10 mM Ca^{2+}; T_H = 5–23 °C. (4) Subsequent cooling scan of sample 3; T_H = 6–22 °C.

A second heating scan after the first heating–cooling cycle produced a curve similar to the first heating scan. Further cycling did not alter the characteristics of either curve.

Hexagonal Transitions in Other Lipids. There are lipids other than PE which will undergo a bilayer-to-hexagonal transition. Results from X-ray diffraction, ^{31}P NMR, and freeze–fracture electron microscopy have shown that the sodium salts of cardiolipins form bilayers, while divalent cation salts of cardiolipins are capable of forming hexagonal structures (Rand & Sengupta, 1972; Vasilenko et al., 1982). The temperature of this transition is dependent on the divalent cation used and the fatty acid composition of the cardiolipin. When bovine heart or *E. coli* cardiolipin SUV at pH 7.4 were injected with concentrated Ca^{2+} solution (one to two Ca^{2+} ions per cardiolipin), the lipid aggregated. These Ca^{2+}–cardiolipin aggregates did not show any transition when probed by 0.2 mol % NBD-PE in the continuous temperature scan above 0 °C. This is consistent with the results obtained by X-ray diffraction, indicating that Ca^{2+} precipitates of bovine heart cardiolipin are in hexagonal phase even at relatively low temperatures (Rand & Sengupta, 1972). In fact, ^{31}P NMR studies on the Ca^{2+}–cardiolipin complex in the presence of 30% glycerol have shown that the bilayer-to-hexagonal transition is around −10 °C (Vasilenko et al., 1982).

The addition of PC to PE stabilizes the bilayer structure in phospholipid dispersions (Cullis & de Kruijff, 1979). The effect of PC on the Ca^{2+}–cardiolipin complex was examined by the same fluorometric method. Figure 8 shows the continuous temperature scans of the fluorescence intensity of NBD-PE-labeled cardiolipin–PC (1:1 mole ratio) preaggregated by addition of Ca^{2+} (equimolar to cardiolipin). Both *E. coli* cardiolipin–PC and bovine heart cardiolipin–PC showed broad transitions at 30–40 and 5–15 °C, respectively, while the transitions of pure cardiolipin–Ca^{2+} complex occur at much lower temperature. The transition of either system is very reproducible; it can be scanned repetitively through heating–cooling cycles, and the transition recurs at the same temperature region.

Discussion

We have presented a new method for studying the bilayer-to-hexagonal phase transition. This method can be used not only on the aggregated lipid samples usually used with other methods but also on dilute suspensions of liposomes. The most direct indication that the intensity of NBD-PE fluorescence can be used for detecting the bilayer-to-hexagonal phase transition was that there was a good correlation between the transition temperature obtained by DSC on various PE samples and the temperature region, with a discontinuous increase of fluorescence intensity in the fluorometric heating scan of similar samples of egg PE, which had been preaggregated by protons or calcium (Figure 3). Further study revealed that the temperature at which NBD-PE fluorescence increased significantly in response to added ions in a dilute liposome suspension correlated very well with the onset of the bilayer-to-hexagonal phase transition, as shown in Figure 4 and Table I. Experiments of the latter type have been recently used for determining the onset of the transition (Ellens et al., 1986). With our method, we are able to determine both the onset and end point of the bilayer-to-hexagonal phase transition for a dilute suspension of liposomes. It is important to emphasize that this method, unlike X-ray diffraction and freeze–fracture electron microscopy, does not offer structural information and therefore cannot be used to identify the existing hexagonal phase in membranes with unknown phase behavior. If there is no phase change, our method cannot be used to determine membranes phases, either in single or in mixed phases.

One can generally learn about the environment surrounding a fluorophore from an examination of its emission spectra. The emission maximum and the relative quantum yield are two factors which are affected by the probe's environment. It has been shown previously [e.g., see Waggoner and Stryer (1970)] that the relative quantum yield increases and the emission maximum shifts to shorter wavelength when a probe goes to

a less polar or less hydrated environment. Since NBD is attached to the headgroup of PE, it is most likely to be sensitive to changes of the bilayer surface and the headgroup region (Chattopadhyay & London, 1986). As demonstrated in the experiments on liposomes of egg PC or DMPC, NBD-PE does not respond to the changes of the ionic conditions in the bulk media.

Since NBD-PE represents only a minor impurity in the lipid of interest, there is a possibility that the probe molecules may phase-separate from the major lipids. Such a phase separation will increase the local concentration of NBD-PE and lead to fluorescence self-quenching. This phenomenon is observed when lipids containing 1 mol % NBD-PE are in gel phase (e.g., egg PE near 0 °C and DMPC below 10 °C). Fluorescence self-quenching is avoidable when 0.1 mol % probe is used. Most of our present study has been done with 0.1 mol % NBD-PE. It should be pointed out that *an increase* of the fluorescence intensity is seen in the temperature region where the bilayer-to-hexagonal transition occurs even in PE liposomes containing NBD-PE up to 1 mol %, as opposed to *a decrease* due to phase separation of probe molecules.

In order to further characterize this new method, we have examined various lipids known to undergo bilayer-to-hexagonal transition triggered either thermotropically or ionotropically. Since the polymorphism of PE has been well studied by other methods, we have examined PE more extensively, and results are consistent with the published data. The aggregated PE prepared by dialyzing MLV prepared at pH 9.5 against low pH or Ca^{2+} solution represents an equilibrated system which consistently shows reproducible heating and cooling scans with clearly defined transition points. We also measured the transition in a dilute lipid suspension and have plotted the change of fluorescence intensity in response to the ion addition as a function of temperature. Even though this type of experiment is more time-consuming for obtaining more precisely the onset and end point of the transition, there is the added advantage that it can provide kinetic information about the transformation from bilayer-to-hexagonal phase in membrane systems which are initially in dilute suspension.

It has been shown that liposomes composed of PE become highly permeable to ions and other water-soluble molecules at the temperature of the hexagonal transition (Ellens et al., 1986). This is an additional parameter that could have influenced our results. Thus, Ca^{2+} or H^+ could be excluded from the interior of MLV at temperature below the transition to hexagonal, and therefore not available to interact with the majority of the PE groups located on the interior bilayers of MLV. However, this possibility is excluded by the reproducible heating and cooling scans of the preaggregated samples, by the length of time allowed for equilibration during dialysis of the samples, by the expected relatively high permeability of these bilayers toward H^+ and Ca^{2+} (Ellens et al., 1986), and by comparable results obtained by the two methods we have employed: heating–cooling scans of preaggregated samples, or addition of ions to dilute liposome suspensions at different temperatures. Our conclusion is therefore that the increase in fluorescence we observe is related to the formation of hexagonal phase (which is promoted by H^+ or Ca^{2+} at certain temperatures) and not by the mere titration of PE headgroups by either H^+ or Ca^{2+}.

In general, lipids in hexagonal phase are highly aggregated so that the possibility that the light scattering interferes with the fluorescence measurement had to be considered. Figure 4 addresses this concern and demonstrates that the observed changes in the fluorescence at 530 nm are not a result of the lipid aggregation. In principle, the maximum fluorescence intensity observed after ion addition should represent the fluorescence of the equilibrium state under the new ionic conditions, and once this maximum has been reached, the intensity should stay at that level. Following the time course of the actual fluorescence intensity, one can estimate the rate of the hexagonal phase formation in dilute liposome suspensions. It should be noted that a slow decrease in fluorescence intensity is observed at later times after ion addition. This is a result of extensive aggregation (clumping) of the sample and the adhesion of macroscopically visible clumps to the cuvette walls and the magnetic stirbar. Since the decline in fluorescence due to such "clumping" occurs relatively slowly compared to the initial increase in fluorescence, the fluorescence maximum is affected only slightly and only at higher temperatures where a very rapid transition occurs. This decrease in fluorescence at the latter stage is considered as an after-effect of transition and is more pronounced in Ca^{2+}-induced phase changes.

There are many agents known to lower or raise the bilayer-to-hexagonal transition temperature of PE (Epand, 1985). We have selected two cases to demonstrate that NBD-PE as a probe is useful in measuring those effects on the phase transition. The first additive, deoxycholate, is a detergent which is known to stabilize a bilayer organization for egg PE (Madden & Cullis, 1982). Our measurements indicate that 5 mol % deoxycholate raises the bilayer-to-hexagonal transition of egg PE at pH 5.0 by 5–6 °C. Since only one transition is observed in this detergent–PE system and the transition is not broadened, we conclude that 5 mol % deoxycholate is homogeneously intercalated in the bilayers. Since the pK of deoxycholic acid is 6.58, almost all the deoxycholate molecules are protonated at pH 5.0. Therefore, the effect of deoxycholate on egg PE at pH 5.0 is not likely due to the charge of the ionized carboxyl group. Instead, a disruption of the tight intermolecular hydrogen bonding between PE headgroups by intercalating deoxycholic acid may be the cause of the elevation of the transition temperature.

The second additive, BHT, is a bulky nonpolar compound which lowers the transition temperatures (both the gel-to-liquid-crystalline and the bilayer-to-hexagonal transitions) as shown in Figure 7. A similar effect of BHT on both transitions on TPE has been reported recently using ^{31}P NMR, DSC, and freeze-fracture electron microscopy (Cheng et al., 1986). On the basis of their ^2H NMR study of BHT/perdeuteriated PC mixtures, Cheng et al. (1986) suggest that BHT perturbs preferentially the upper position of the acyl chains (near the glycerol backbone) of the phospholipid. Therefore, the effect of BHT on the bilayer-to-hexagonal transition of egg PE may be due to two factors: dehydration of the bilayer surface and the expansion of the hydrophobic domain of the bilayer due to the nonpolar nature of BHT. In contrast to the sharp transition of the deoxycholate–egg PE system, a broad transition of egg PE–BHT in the heating scan is seen, indicating a less cooperative transition. There is no evidence that more than one transition exists in the system. The high-temperature end of the transition is still 5 °C lower than the transition onset of the control. Even though the heating and cooling scans show different breadths for the transition, it is consistently reproducible when the sample is subjected to further heating and cooling cycles.

We were not able to detect the transition of Ca^{2+} precipitates of cardiolipin from either bovine heart or *E. coli* above 0 °C by the continuous scan setup. These cardiolipin–Ca^{2+} aggregates are probably in hexagonal phase above 0 °C. This

reflects a shortcoming of the fluorometric method which cannot be used to identify absolutely a certain phase. It is likely that the Ca^{2+} salts of both cardiolipins undergo the bilayer-to-hexagonal transitions below 0 °C, which is as low as we could go with our method.

Lipidic particles have been found in cardiolipin–egg PC in the presence of Ca^{2+}, even though the existence of lipidic particles depends highly on technical factors such as cryoprotectants and time of sample processing (Bearer et al., 1982), in addition to experimental conditions such as temperature, divalent cations, pH, and ionic strength [see Verkleij (1984)]. The observation shown in Figure 8 that the fluorescence intensity of NBD-PE in the cardiolipin–PC–Ca^{2+} complex shows a similar response to a temperature scan as is seen in the PE system leads us to regard this as the bilayer-to-hexagonal transition seen by other methods (de Kruijff et al., 1982). This is consistent with the observation that 50 mol % PC can be incorporated into hexagonal phase without any phase separation (Borovjagin et al., 1982). The transitions of both bovine heart cardiolipin–PC–Ca^{2+} and *E. coli* cardiolipin–PC–Ca^{2+} are reproducible in repetitive scans. At present, it is not possible to state whether the lipidic particle structure makes a significant contribution to the transition in the cardiolipin–PC–Ca^{2+} complex observed by our fluorometric method.

In developing this method, we relied heavily on the use of egg PE which is known to undergo hexagonal transition at a convenient temperature range (30–40 °C). We therefore use DSC for an independent determination of the transition temperatures of the same lipid samples as studied by the new fluorometric method. Since the bilayer-to-hexagonal transition is highly dependent on the chain composition, it is difficult to compare transition temperatures directly among publications. As presented in Figure 3, we have used the same batch of egg PE for all the measurements. The hysteresis (i.e., the difference in the phase transition between heating and cooling scans) of preaggregated egg PE at pH 5.0 is larger than that of the Ca^{2+}-induced transition at pH 9.5. It appears that NBD-PE reduces the hysteresis by lowering the transition in the heating scans. A slight difference in transition measured by different methods in our experiments may be explained by one (or more) of the following. Since the fluorophore is located near the headgroup region of the bilayer, it is possible that the probe detects a change in that region of the bilayer more sensitively than the bulk structural rearrangement detected by DSC or other methods. A slightly lower transition temperature would also be reported by the fluorometric method if the probe induced local perturbations favoring the hexagonal phase.

Several factors have been known to be of importance in determining whether a lipid system will undergo the bilayer-to-hexagonal transition. These include the shape of the molecule, the degree of hydrogen bonding of the headgroup, and the hydration of the headgroup (Cullis & de Kruijff, 1979; Seddon et al., 1983). These are not mutually independent. For instance, the PC head group is bulky and more hydrated than PE and does not form intermolecular hydrogen bonding. The binding of Ca^{2+} to PE at high pH or to cardiolipin at neutral pH will condense the headgroup by neutralizing the phosphate group, while also dehydrating the bilayer surface. The Ca^{2+} binding and subsequent vesicle aggregation do not change the fluorescence intensity of NBD until the temperature is up to the bilayer-to-hexagonal transition. This is shown in Figure 6B where $\Delta F = 0$ over the temperature range below the bilayer-to-hexagonal transition. In contrast, there is a small spontaneous increase of fluorescence of NBD when the $-NH_2$ of PE is protonated by lowering the pH from 9.5 to 5.0 at temperatures below the transition ($\Delta F > 0$; Figure 6A). Since we know that lowering the pH does not affect NBD fluorescence directly as seen in egg PC and DMPC, we conclude the protonation of $-NH_2$ of PE is the cause of the slight increase of NBD fluorescence intensity. The exact correlation of protonation of the $-NH_2$ group of PE with NBD fluorescence intensity remains to be studied. Nevertheless, it is clear that NBD is very sensitive to environmental changes of the headgroup region of the bilayers. Furthermore, with the information we have obtained so far, it appears that NBD-PE consistently responds with a relatively high increase in fluorescence intensity whenever any of the above-mentioned factors, such as hydrogen bonding, molecular shape, or dehydration, is affected in such a way that the bilayer-to-hexagonal transition is favored.

In conclusion, we have presented a new method for determining the bilayer-to-hexagonal phase transition. This method can be used on the aggregated lipid samples that have typically been studied with other methods. Besides giving comparable results, this method can provide a clear onset and end point of the transition. The new method has the added advantage that it can also be used to study dilute suspensions of liposomes. This is especially important when one of the components of the system under study can partition between lipid and aqueous phases, e.g., fatty acids (Baldwin et al., 1985; Düzgüneş et al., 1985). Finally, it is possible that this method can be used to measure the rate of transformation from one lipid phase to another in response to added ions or membrane-active compounds at a given temperature. Since the rate can be determined in dilute liposome suspensions, this information can be directly compared to information about liposome aggregation and fusion, and leakage of liposome contents which can be obtained under similar experimental conditions (Wilschut et al., 1980; Ellens et al., 1984; Struck et al., 1981). This approach may provide valuable information about the relevance of the formation of nonbilayer structures to these membrane processes. In addition to the headgroup-labeled NBD-PE studies presented here, other fluorescent probes may be useful for detecting the bilayer-to-hexagonal transition as long as the probe is located near the bilayer surface. In fact, our preliminary results suggest that NBD-PE with the NBD group attached to the methyl end of a lauryl chain can also detect the bilayer-to-hexagonal transition of egg PE possibly because the NBD group bends back into the bilayer surface (Chattopadhyay & London, 1986). In addition, another well-studied probe, dansylphosphatidylethanolamine (Faucon & Lussan, 1973; Waggoner & Stryer, 1970), also responds to the bilayer-to-hexagonal transition of a lipid in a similar manner as NBD-PE (Baldwin et al., 1986).

ACKNOWLEDGMENTS

We thank Drs. P. K. Wolber and N. Düzgüneş for valuable scientific discussions, Dr. S. Murray for editorial comments, and Dr. S. Helgerson for loan of short path-length cuvettes.

REFERENCES

Baldwin, P. A., Düzgüneş, N., & Papahadjopoulos, D. (1985) *Biophys. J. 47*, 112a.

Baldwin, P. A., Hong, K., & Papahadjopoulos, D. (1986) *Biophys. J. 49*, 308a.

Bangham, A. D., Hill, M. W., & Miller, N. G. (1974) *Methods Membr. Biol. 1*, 1–68.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Bearer, E. L., Düzgüneş, N., Friend, D. S., & Papahadjopoulos, D. (1982) *Biochim. Biophys. Acta 693*, 93–98.

Borovjagin, V. L., Vergara, J. A., & McIntosh, T. J. (1982) *J. Membr. Biol. 69*, 199–212.
Casal, H. L., & Mantsch, H. H. (1984) *Biochim. Biophys. Acta 779*, 381–401.
Chattopadhyay, A., & London, E. (1986) *Biophys. J. 49*, 308a.
Cheng, K.-H., Yeagle, P. L., Lepock, J. R., & Hui, S. W. (1986) *Biophys. J. 49*, 324a.
Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.
De Kruijff, B., Verkleij, A. J., Leunissen-Bijvelt, J., van Echteld, C. J. A., Hille, J., & Rijnbout, H. (1982) *Biochim. Biophys. Acta 693*, 1–12.
Düzgüneş, N., Straubinger, R. M., Baldwin, P. A., Friend, D. S., & Papahadjopoulos, D. (1985) *Biochemistry 24*, 3091–3098.
Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532–1538.
Ellens, H., Bentz, J., & Szoka, F. C. (1986) *Biochemistry 25*, 285–294.
Epand, R. M. (1985) *Biochemistry 24*, 7092–7095.
Faucon, J.-F., & Lussan, C. (1973) *Biochim. Biophys. Acta 307*, 459–466.
Harlos, K., & Eibl, H. (1981) *Biochemistry 20*, 2858–2892.
Luzzati, V. (1968) in *Biological Membranes* (Chapman, D., Ed.) Vol. 1, pp 71–123, Academic, London.
Madden, T. D., & Cullis, P. R. (1982) *Biochim. Biophys. Acta 684*, 149–153.
Rand, R. P., & Sengupta, S. (1972) *Biochim. Biophys. Acta 255*, 484–492.
Seddon, J. M., Cevc, G., & Marsh, D. (1983) *Biochemistry 22*, 1280–1289.
Shinitzky, M., & Barenholz, Y. (1978) *Biochim. Biophys. Acta 515*, 367–394.
Sklar, L. A., Hudson, B. S., & Simoni, R. D. (1977) *Biochemistry 16*, 819–828.
Struck, D., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.
Vasilenko, I., De Kruijff, B., & Verkleij, A. J. (1982) *Biochim. Biophys. Acta 684*, 282–286.
Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43–63.
Waggoner, A. S., & Stryer, L. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 579–589.
Wilschut, J., Düzgüneş, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.

Isolation and Characterization of a Complementary DNA for the Nuclear-Coded Precursor of the β-Subunit of Bovine Mitochondrial F_1-ATPase†

Gail A. M. Breen,* Priscilla L. Holmans, and Karen E. Garnett

Molecular and Cell Biology Program, The University of Texas at Dallas, P.O. Box 830688, Richardson, Texas 75083-0688

Received October 8, 1987; Revised Manuscript Received January 29, 1988

ABSTRACT: We have isolated a cDNA clone encoding the precursor of the β-subunit of the bovine heart mitochondrial F_1-ATPase. Two probes were used to isolate this precursor from a bovine heart cDNA library. One probe was a mixed-sequence oligonucleotide directed against a portion of the amino acid sequence of the mature protein, and the other probe was the F_1-ATPase β-subunit gene from *Saccharomyces cerevisiae*. Determination of the nucleotide sequence of this cDNA reveals that it contains a 1584-nucleotide-long open reading frame that encodes the complete mature β-subunit protein and a 48 amino acid long NH_2-terminal extension. This amino-terminal presequence contains four basic arginine residues, one acidic glutamic acid residue, four polar uncharged serine residues, and five proline residues. Southern blot hybridization analyses suggest that the bovine F_1-ATPase β-subunit precursor is encoded by a single genetic locus. RNA blot hybridization analyses reveal a single mRNA species of approximately 1.9 kilobases from both bovine liver and heart.

The mitochondrial ATP synthase complex synthesizes ATP from ADP and P_i, utilizing energy from the electron-transport chain. This complex consists of two distinct regions: a soluble F_1 region that contains the catalytic site for ATP synthesis and a membrane-integrated F_0 region that is involved in proton translocation [for reviews, see Amzel and Pedersen (1983), Futai and Kanazawa (1983), and Senior and Wise (1983)]. The ATP synthase complex, like several of the other complexes of the mitochondrial oxidative phosphorylation system, is composed of proteins encoded by two distinct genetic systems. For example, two of the subunits of the F_0 region of the mammalian ATP synthase complex are encoded by the mitochondrial DNA and translated on ribosomes within the mitochondria (Anderson et al., 1981; Bibb et al., 1981; Fearnley & Walker, 1986). The rest of the subunits are encoded by nuclear genes, synthesized on cytoplasmic ribosomes (often as larger precursors), imported into mitochondria, processed to give the mature subunits, and then assembled together with the other subunits to form a functional complex [for reviews, see Tzagoloff and Myers (1986) and Douglas et al. (1986)]. The molecular mechanisms that govern the expression of the nuclear genes that encode proteins of the mitochondrial oxidative phosphorylation system and their coordination with the mitochondrial genes are largely unknown. Furthermore, the sequences required for the import and sorting of these proteins to the mitochondrion have not

† This work was supported by a grant from the National Institutes of Health.
* Address correspondence to this author.

0006-2960/88/0427-3955$01.50/0