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Membrane Contact, Fusion, and Hexagonal (H_{II}) Transitions in Phosphatidylethanolamine Liposomes†

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ABSTRACT: The behavior of phosphatidylethanolamine (PE) liposomes has been studied as a function of temperature, pH, ionic strength, lipid concentration, liposome size, and divalent cation concentration by differential scanning calorimetry (DSC), by light scattering, by assays measuring liposomal lipid mixing, contents mixing, and contents leakage, and by a new fluorometric assay for hexagonal (H_{II}) transitions. Liposomes were either small or large unilamellar, or multilamellar. Stable (impermeable, nonaggregating) liposomes of egg PE (EPE) could be formed in isotonic saline (NaCl) only at high pH (>8) or at lower pH in the presence of low ionic strength saline (less than 50 mOsm). Bilayer to hexagonal (H_{II}) phase transitions and gel to liquid-crystalline transitions of centrifuged multilamellar liposomes were both detectable by DSC only at pH 7.4 and below. The H_{II} transition temperature increased, and the transition enthalpy decreased, as the pH was raised above 7.4, and it disappeared above pH 8.3 where PE is sufficiently negatively charged. H_{II} transitions could be detected at high pH following the addition of Ca^{2+} or Mg^{2+}. No changes in light scattering and no lipid mixing, mixing of contents, or leakage of contents were noted for EPE liposomes under nonaggregating conditions (pH 9.2 and 100 mM Na^+ or pH 7.4 and 5 mM Na^+) as the temperature was raised through the H_{II} transition region. However, when aggregation of the liposomes was induced by addition of Ca^{2+} or Mg^{2+}, or by increasing [Na^+], it produced sharp increases in light scattering and in leakage of contents and also changes in fluorescent probe behavior in the region of the H_{II} transition temperature (T_H). Lipid mixing and contents mixing were also observed below T_H under conditions where liposomes were induced to aggregate, but without any appreciable leakage of contents. We conclude that H_{II} transitions do not occur in liposomes under conditions where intermembrane contacts do not take place. Moreover, fusion of PE liposomes at a temperature below T_H can be triggered by H^+, Na^+, Ca^{2+}, or Mg^{2+} or by centrifugation under conditions that induce membrane contact. There was no evidence for the participation of H_{II} transitions in these fusion events.

The ability of phosphatidylethanolamine and other phospholipids to assume nonbilayer configurations has been well documented. Evidence for the occurrence of hexagonal (H_{II}) phases in phospholipids comes from X-ray diffraction (Luzzati et al., 1968; Rand et al., 1971; Harlos & Eibl, 1981; Gruner et al., 1988), ^{31}P NMR studies (Cullis & de Kruijff, 1978, 1979; Hui et al., 1981), freeze-fracture electron microscopy (Deamer et al., 1970; de Kruijff et al., 1979; van Venetie & Verkleij, 1981), infrared spectroscopy (Mantsch et al., 1981), electron spin resonance spectroscopy (Hardman, 1982), and differential scanning calorimetry (Harlos & Eibl, 1981; Seddon et al., 1983). The evidence for hexagonal (H_{II}) phases for phosphatidylethanolamine comes primarily from studies in which multibilayers of phospholipid have been studied at very high phospholipid concentrations. These conditions allow for the presence of large areas of bilayer in close proximity, and may favor formation of nonbilayer lipid under the appropriate thermodynamic conditions given the extensive three-dimensional character of the H_{II} phase.

Nonbilayer lipid has been postulated to play an important biological role in cell membranes particularly in fusion and transport functions (Hope & Cullis, 1981; Cullis et al., 1980; Venetie & Verkleij, 1982; Ellens et al., 1989). However, suspensions of phospholipid vesicles, or liposomes, are a closer approximation to biological membranes than are closely packed multilayers. Therefore, a study of unilamellar liposomes under conditions leading to nonbilayer structures or H_{II} transitions should provide us with relevant information about the occurrence and role of nonbilayer lipid in biological membranes.

The difficulty in forming stable liposomes of phosphatidylethanolamine (PE)[1] at physiological pH in isotonic buffers has

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[1] Abbreviations: PE, phosphatidylethanolamine; EPE, egg yolk PE; TPE, PE prepared from egg phosphatidylcholine by transesterification; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium bromide); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DSC, differential scanning calorimetry; SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s); MLV, multilamellar vesicle(s); T_c, gel to liquid-crystalline phase transition temperature; T_H, liquid-crystalline to hexagonal (H_{II}) phase transition temperature.

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been reported previously (Papahadjopoulos & Miller, 1967). These authors had observed that raising the pH to deprotonate the amino group had pronounced effects on the morphology and stability of the suspensions. Stable small unilamellar vesicles (SUV) composed of PE have been prepared at low ionic strength above pH 9.0 (Stollery & Vail, 1977; Kolber & Haynes, 1979). Lowering the pH of these vesicles down to physiological levels by dialysis did not alter vesicle morphology (Stollery & Vail, 1977). More recent studies with large unilamellar vesicles (LUV) composed of various PE and prepared at high pH have reported on their aggregation, fusion, or lysis upon acidification or addition of divalent metals (Ellens et al., 1986, 1989). Generally, a correlation has been observed between aggregation, destabilization of the vesicles, and the occurrence of the hexagonal phase transition in multilamellar vesicles (MLV).

For this study, we prepared small (SUV) and large (LUV) unilamellar vesicles and multilamellar vesicles (MLV) of egg PE (EPE) and of PE transesterified from egg phosphatidylcholine (TPE). Using these vesicles, we examined the effect of temperature, pH, ionic strength, lipid concentration, and divalent cations on mixing of lipids and contents between liposome populations, and on leakage of contents. Light scattering and differential scanning calorimetry were used to monitor the occurrence of aggregation and lipid phase transitions. We have attempted to analyze the differences in behavior between MLV and SUV or LUV and to relate membrane fusion with phase behavior using similar types of vesicles.

Materials and Methods

Egg phosphatidylethanolamine (EPE), PE prepared by transesterification from egg phosphatidylcholine (TPE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). All lipids were chromatographically pure by thin-layer chromatography on silica gel in $CHCl_3$/MeOH/acetone/acetic acid/H_2O (5:1:2:1:0.5). 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes, Inc. (Eugene, OR).

PE liposomes (SUV and MLV) were prepared as follows. Chloroform solutions of EPE or TPE containing 10 μmol of phospholipid, and fluorescent phospholipids if desired, were evaporated to dryness under vacuum. For liposomes at high pH in isotonic buffer, 1 mL of 10 mM Tris buffer containing 100 mM NaCl and 0.1 mM EDTA, pH 9.2, was added to the lipids. After a brief period of vortexing under an argon atmosphere in a capped glass container, the pH was checked. It was usually on the acid side, and PE does not hydrate well under these conditions. The pH was adjusted with small additions of 0.04 N NaOH, with vortexing under argon in between additions to aid in hydration of the PE, until the pH reached 9.2 and the liposomes formed a homogeneous suspension with no evidence of flocculation or aggregation. Either this suspension was used immediately as MLV or it was extruded through Nucleopore polycarbonate membranes (Olson et al., 1979) with pores of 0.05–0.08-μm diameter under a nitrogen pressure of approximately 20–200 psi to give liposomes of narrower size ranges. Such preparations although referred in the text as MLV may contain substantial amounts of oligolamellar vesicles, especially after extrusion through polycarbonate membranes. SUV were formed by sonicating the MLV suspension to a clear or slightly opalescent solution (15–30 min) in a bath-type sonicator (Laboratory Supplies Ltd., Hicksville, NY) in a capped glass test tube under argon atmosphere at 20 °C.

PE liposomes at physiological pH and low ionic strength were formed by addition of 10 mM TES buffer containing 5 mM NaCl and 0.1 mM EDTA, pH 7.4, to dried phospholipid and vortexing under argon. Again, it was necessary to adjust the pH to 7.4 with 0.04 N NaOH in order to achieve a homogeneous phospholipid suspension. Hydration was aided by bringing the pH above 7.4 and returning it to 7.4 by dialysis against pH 7.4 buffer when the phospholipid was fully hydrated. We have found some variation between different batches of EPE and TPE, and for one lot of EPE, it was necessary to raise the pH above 7.9 before a hydrated, nonaggregating suspension of liposomes could be obtained at low ionic strength. The extrusion and sonication procedures for MLV and SUV of low ionic strength liposomes were as described above.

LUV of PE were prepared by the reverse-phase evaporation procedure of Szoka and Papahadjopoulos (1978) with the following modification: A 0.34-mL aqueous suspension of PE was prepared and adjusted to the desired pH as described above. To this suspension was added 1 mL of ether, the mixture was sonicated under argon to form a suspension, and LUV were made as previously described. The liposomes were extruded through 0.1-μm polycarbonate membranes (Nucleopore) at a nitrogen pressure of 80 psi (Olson et al., 1979).

The suspension was then centrifuged for 20 min at 10000*g* for removing any remaining large particles. Such a procedure usually produces liposomes with more than 90% of the vesicle population in the range of 0.1–0.15-μm diameter (Szoka et al., 1980).

All liposomes (SUV, MLV, and LUV) which contained encapsulated material (see below) were separated from nonencapsulated material by chromatography on Sephadex G-75 (Pharmacia). Phospholipid concentrations were determined according to Bartlett (1959). Most experiments were done at a lipid concentration of 50 μM, but in experiments involving DSC (see below), and in some fluorescence experiments, higher concentrations of liposomes were used.

Lipid mixing assays were done by a modification of the method of Hoekstra (1982) or by modifications of the method of Struck et al. (1981). In the first method, 1.3 mol % of NBD-PE (donor) was included in one population of liposomes and 3.4 mol % of Rh-PE (acceptor) was included in a second population of liposomes (probe quenching method). Lipid mixing between the two populations resulted in a decrease in the relative NBD fluorescence due to resonance energy transfer between the two probes. Upon complete mixing of the two probes, relative NBD fluorescence levels declined to 4% of maximum. In the second method, 0.15 mol % of NBD-PE and 0.42 mol % of Rh-PE were included in one population of liposomes, and these were interacted with a second population of liposomes containing no probe (probe dilution method). Lipid mixing between the two populations resulted in an increase in the NBD fluorescence as the distance between the two probes increased. Maximum NBD fluorescence was determined by incorporating appropriate amounts of NBD-PE and Rh-PE together in a third liposome population. In the lipid mixing assays, we have found that the NBD fluorescence is less sensitive to perturbation by Ca^{2+} when the acceptor molecule is present in excess (Düzgüneş et al., 1987). The fluorescence emission of NBD was monitored by using an SLM-4000 spectrofluorometer at an excitation wavelength of 475 nm and an emission wavelength of 530 nm. A stirring apparatus located beneath the temperature-controlled cuvette

holder enabled constant mixing of cuvette contents.

Leakage of contents from PE liposomes was measured by the calcein dequenching method of Allen and Cleland (1980). Calcein at a concentration of 50 mM (200 mOsm) in 10 mM Tris, pH 9.2, was trapped in the high-pH liposomes, and calcein at a concentration of 20 mM (80 mOsm) in 10 mM TES buffer, pH 7.4, was trapped in the low osmotic strength liposomes. Maximum fluorescence was determined by treating the samples with 50 μL of 10% Triton X-100. Calcein was excited at 430 nm, and fluorescence (>530 nm) was monitored by using a Corning 3-68 cutoff filter.

Mixing of aqueous contents (fusion) was determined in PE liposomes by the ANTS/DPX method of Ellens et al. (1984) with the following modification. In one population of liposomes, 10 mM ANTS, pH 8.3 (50 mOsm), was entrapped, and 15 mM DPX, pH 8.3 (50 mOsm), was entrapped in a second liposome population. These lower concentrations of compounds allowed us to keep the ionic strength of the solutions below the ionic strength where aggregation occurred in the PE liposomes used in these experiments. Mixing of aqueous contents leads to a decrease in fluorescence as ANTS fluorescence is quenched by DPX. ANTS fluorescence (>530 nm) was followed by means of a Corning 3-68 cutoff filter, with the excitation wavelength at 390 nm.

Light-scattering experiments were performed at the same time as calcein leakage, lipid mixing, or contents mixing using the second channel of the SLM-4000 spectrofluorometer at 430, 475, or 390 nm, respectively. Some light-scattering measurements were performed independently with excitation and emission wavelengths set at 276 nm.

Differential scanning calorimetry experiments were performed on a Perkin-Elmer DSC-2 instrument. The transition temperatures and enthalpies were calibrated with indium. Five to seven micromoles of PE (MLV) which had been concentrated by centrifugation at 10000*g* in an Eppendorf microcentrifuge was placed into aluminum pans which had been cleaned by washing in $CHCl_3$/MeOH, 2:1, and heating to 400 °C. The concentration of lipid in the pelleted suspension was approximately 0.3 mol/L. For some suspensions which were not aggregated, the centrifugation was at 1000000*g* for at least 3 h. The reference pan contained buffer at the same pH and osmotic strength as that in the liposomes. Volume in the sample and reference pans was 15 μL. The samples were equilibrated for at least 10 min and were scanned through three or more heating and cooling scans at a rate of 5 °C/min. The transition temperatures are reported as peak values (the intercept of the tangent of the rising slope with the tangent of the descending slope). After the samples were scanned, the pans were opened, the lipid was dissolved in 0.2% Triton X-100, and the total phosphate was determined by the method of Bartlett (1959). The enthalpies were calculated from the peak areas which were determined by weight.

High-sensitivity thermograms were taken on a Microcal MC-2 scanning calorimeter (Microcal, Inc., Amherst, MA). Egg PE liposomes were prepared with 0.1% NBD-PE by either the MLV or the LUV methods. A pH 9.0 buffer (10 mM Trizma/5 mM NaCl) was used in the liposome preparations with final lipid concentrations of 20 mM in both procedures. The liposomes were extruded 10 times through appropriate pore sizes of Nucleopore polycarbonate filters. The pH of the liposomes was lowered by dialysis against a pH 7.4 buffer (10 mM TES/5 mM NaCl) overnight at 4 °C. A 1.2-mL sample was used at a concentration of 10–20 mM lipid for each DSC run, at a scan rate of 0.76 °C/min. Two thermograms were obtained for each sample, with no noticeable differences between them. Reproducibility was judged to be well within a 5% limit.

Table I: DSC of PE (MLV) as a Function of pH, $[Na^+]$, or Divalent Cation Concentration

lipid	pH	[NaCl] (mM)	$[Ca^{2+}]$ (mM)	$[Mg^{2+}]$ (mM)	T_c (°C)	T_H (°C)
EPE	7.4	5	0	0	9	46
EPE	9.2	100	0	0	9	
EPE	7.4	5	3	0	9	38
EPE	7.4	5	0	3	9	40
EPE	9.2	100	7	0	9	38
EPE	9.2	100	0	7	9	48

Table II: DSC of EPE MLV at Low Ionic Strength as a Function of pH[a]

pH	T_c (°C)	ΔH_{T_c} (kcal/mol)	T_H (°C)	ΔH_{T_H} (kcal/mol)
7.4	12.5	3.0	42.5	0.7
8.0	12.0	4.7	44.0	0.5
8.3	12.0	4.2	47.5	0.3
8.4	12.0	2.9	ND[b]	0.0
8.6	12.0	3.9	ND	0.0
9.2	12.0	4.2	ND	0.0

[a] T_c and T_H are given as peak values. The temperature range of the H_{II} transition did not change significantly with increasing pH. [b] ND, not detectable.

Results

DSC Studies. Differential scanning calorimetry of EPE (MLV) suspended in 5 mM NaCl at pH 7.4 detected a gel to liquid-crystalline transition (T_c) at 9 °C and a hexagonal (H_{II}) transition (T_H) at 46 °C (Table I). For TPE, the respective transition temperatures were 17 and 58 °C (not shown). Raising the pH to 9.2 (100 mM NaCl) caused disappearance of the H_{II} transition with no evidence of T_H at temperatures up to 80 °C. No change was observed in T_c at higher pH. The disappearance of T_H at high pH is likely due to charge repulsion between adjacent membranes under conditions where the amino group is deprotonated and PE carries a net negative charge. This would not allow for the close approach of the membranes which may be necessary for the cooperative formation of the hexagonal phase. Addition of Ca^{2+} or Mg^{2+} at a concentration of 3 mM at pH 7.4 (5 mM NaCl) lowered the T_H for EPE (MLV) slightly. Addition of 7 mM Ca^{2+} or Mg^{2+} at pH 9.2 (100 mM NaCl) to EPE preparations resulted in reappearance of the H_{II} transition at a T_H similar to that obtained at pH 7.4 without Ca^{2+}. Mg^{2+} had a tendency to shift the T_H to higher temperatures by a few degrees centigrade at pH 9.2 (Table I). It seems likely that addition of divalent cations overcomes the net negative charge of the bilayer membranes at high pH (probably by binding to the phosphate), and thus promotes aggregation leading to the formation of hexagonal phase. As the pH increases, higher concentrations of divalent cations are needed in order to restore the H_{II} transition. Similar trends were observed for TPE liposomes (not shown).

DSC results for a different lot number of EPE are given in Table II. This batch of PE had a T_c of 12 °C and a T_H of 42 °C, and would form homogeneous suspensions of liposomes only above pH 7.9 in low ionic strength buffer, unlike the previous batch of EPE which readily formed liposomes at pH 7.4 at low ionic strength. It is possible that differences in batches of PE reflect contamination with small amounts of negatively charged lipid which prevent aggregation and flocculation of bilayer suspensions through charge–charge repulsion. However, this was not detectable by thin-layer chromatography. Differences in fatty acid composition or

Table III: High-Sensitivity Thermograms of EPE as a Function of pH and Liposome Size

pH	filter size (μm)	T_c (°C)	ΔH_{T_c} (kcal/mol)	T_H (°C)	ΔH_{T_H} (kcal/mol)
7.4	0.4	10.0	4.1	41.7	0.38
9.0	0.4	10.0	3.6	ND[a]	0.0
7.4	0.05	10.0	2.8	ND	>0.3

[a] ND, not detectable.

oxidation may also be involved. In this preparation of EPE (MLV), as the pH was increased from 7.4 to 8.3, the T_H increased, and the enthalpy of the transition (ΔH) decreased. No H_{II} transition was observed at pH 8.4 and above. We conclude that this preparation of PE aquires sufficient net negative charge at pH 8.4 to prevent the close apposition of membranes, thus inhibiting formation of the hexagonal (H_{II}) phase.

Suspensions of MLV or LUV at higher pH, and when particle size was reduced to 0.1 μm or less, required very high centrifugation speeds to pellet the lipids for detection of phase transitions using the relatively insensitive Perkin-Elmer DSC-2 instrument. We therefore did a series of experiments using a high-sensitivity DSC instrument to examine the effect of pH or particle size on suspensions of EPE liposomes which had much reduced lipid concentrations relative to the pelleted material (Figure 1). Although the gel to liquid-crystalline transition was well delineated in these EPE suspensions with similar T_c values and enthalpies to those of the pelleted samples (Tables II and III), the H_{II} transition was either considerably reduced in enthalpy or absent (Table III).

No H_{II} transition was observed in larger (0.4 μm) LUV at pH 9.0, 5 mM NaCl, indicating that the lack of an H_{II} transition in the pelleted material was not due only to our inability to obtain a sufficiently concentrated sample of liposomes from these preparations (Figure 1A, Table III). However, a clear, albeit small, transition centered at 42 °C was observed in the same preparation of liposomes when the pH was reduced to 7.4, 5 mM NaCl (Figure 1B, Table III). Further reduction in the particle size of the liposome preparations by extrusion through 0.05-μm filters at pH 7.4 caused extensive broadening of the enthalpy of the H_{II} transition (Figure 1C, Table III) to the extent that the presence of the transition or the enthalpy of the transition, if any, could not be determined with any degree of certainty.

Lipid Mixing Studies. Fusion of SUV of PE in the presence of Ca^{2+} or Mg^{2+} has been reported by Stollery and Vail (1977). We have examined initial rates of lipid mixing for SUV liposome populations at 25 °C as a function of Ca^{2+} or Mg^{2+} concentration at pH 7.4, low [Na^+], and at pH 9.2, 100 mM NaCl. NBD-PE was included in one population of vesicles and Rh-PE in the other. In the absence of Ca^{2+} or Mg^{2+}, no lipid mixing was observed. The threshold for Ca^{2+}-induced lipid mixing for SUV (EPE or TPE) was 0.8 mM at pH 7.4 (5 mM NaCl) and increased to approximately 3 mM at pH 9.2 (100 mM NaCl). Thresholds for Mg^{2+}-induced lipid mixing were slightly higher than that for Ca^{2+} (results not shown). The higher concentrations of divalent cations needed to induce lipid mixing of SUV at pH 9.2 may be a reflection of the greater net negative charge carried by PE at higher pH. Initial rates of lipid mixing were determined at different liposome concentrations, and reaction orders of 1.96 and 1.92 were determined for EPE and TPE, respectively (results not shown), indicating that aggregation is the rate-limiting process for lipid mixing.

The initial rates of lipid mixing for SUV and for LUV (extruded through membranes of 0.1-μm pore diameter)

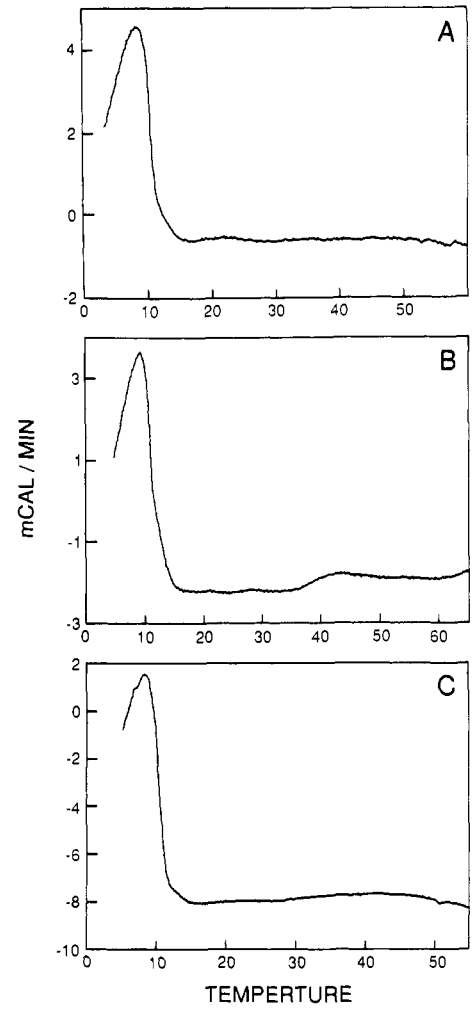

FIGURE 1: High-sensitivity thermograms of EPE LUV were taken on a Microcal scanning calorimeter. (A) Liposomes in pH 9.0, 5 mM NaCl buffer were extruded 10 times through 0.4-μm pore size Nucleopore filters; 1.2 mL of an 11.15 mM phospholipid liposome suspension was used. (B) The same experimental conditions were applied as described in (A) except that liposomes were in pH 7.4, 5 mM NaCl buffer. (C) Liposomes in pH 7.4, 5 mM NaCl buffer were sequentially extruded 10 times through 0.4-, 0.2-, 0.1-, 0.08-, and 0.05-μm Nucleopore filters; 1.2 mL of a 19.65 mM phospholipid suspension was used.

composed of EPE and TPE were studied as a function of temperature, in order to examine whether lipid mixing would be much enhanced at the H_{II} transition temperature if the bilayers underwent reorganization. No evidence of lipid mixing (or alterations in light scattering indicating aggregation) was apparent either at pH 9.2 (100 mM NaCl) or at pH 7.4 (5 mM NaCl) at temperatures up to 60 °C, which is well beyond the T_H for both lipids in the form of MLV as measured by DSC.

We also measured initial rates of lipid mixing (probe quenching method) as a function of temperature for SUV and LUV (extruded through membranes of 0.1-μm pore diameter) of EPE and TPE in the presence of concentrations of Ca^{2+} and Mg^{2+} sufficient to induce aggregation. Under these conditions, liposomes at both pH 7.4 and pH 9.2 showed H_{II} transitions by DSC. The results for LUV (extruded through membranes of 0.1-μm pore diameter) of EPE are shown in Figure 2. Reproducible changes in the slope of initial rates versus temperature were apparent for both SUV (not shown) and LUV in the presence of either Ca^{2+} or Mg^{2+} at both pH 7.4 (5 mM NaCl) (Figure 2) and pH 9.2 (100 mM NaCl) (not shown). A region of depressed initial rates was observed which corresponded to the observed T_H for EPE in the presence

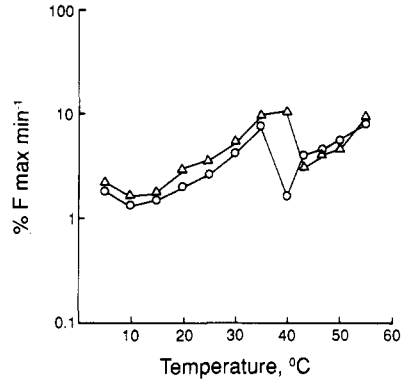

FIGURE 2: Initial rates of lipid mixing (probe quenching method) as a function of temperature for LUV (0.1 μm) of EPE at pH 7.4, 5 mM NaCl. Phospholipid concentration was 50 μM. (○) 0.8 mM Ca^{2+}; (△) 1.0 mM Mg^{2+}.

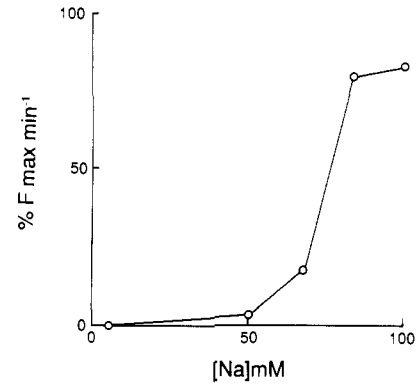

FIGURE 3: Effect of increasing Na^+ concentration on initial rates of lipid mixing (probe quenching method) for EPE, SUV (50 mM); pH 7.4, 5 mM NaCl at 20 °C.

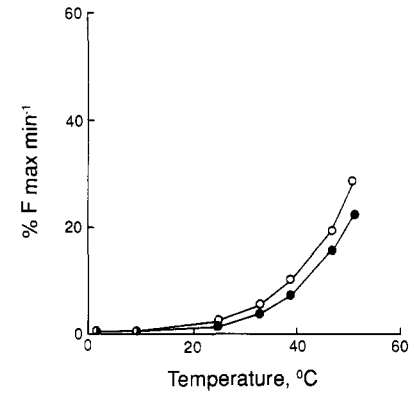

FIGURE 4: Initial rates of leakage of 20 mM calcein as a function of temperature for LUV (0.1 μm, EPE). (○) pH 7.4, 5 mM NaCl, 1 mM Ca^{2+}; (●) pH 9.2, 100 mM NaCl, 3.5 mM Ca^{2+}.

of Ca^{2+} or Mg^{2+} as measured by DSC. Also apparent were depressions in initial rates corresponding to T_c temperatures for EPE (Figure 2). This was confirmed by repeating the measurements at closely spaced temperature points in the T_c region (results not shown). Initial rates of lipid mixing for LUV (0.1 μm) were lower than those seen for SUV at the same ion concentrations and temperatures. Lower lipid mixing rates have also been seen for LUV of phosphatidylserine as compared to SUV (Wilschut et al., 1980; Nir et al., 1983; Düzgüneş et al., 1987). Similar trends were observed for SUV and LUV of TPE (not shown).

Large increases in light scattering, which reflect changes in the aggregation state of the liposomes, were seen for both SUV and LUV of either EPE or TPE in the region of T_H upon addition of Ca^{2+} or Mg^{2+} at both pH 7.4 (5 mM NaCl) and pH 9.2 (100 mM NaCl) (results not shown), but no changes in light scattering were observed in the absence of substances which promote interbilayer contact.

The explanation for the anomalies in the slopes of initial rates in the temperature regions of T_c and T_H lies in changes in the fluorescence properties of the NBD probe at these transitions (Hong et al., 1988). NBD-PE by itself, or in combination with Rh-PE, appears to be useful for detecting T_c or T_H, but the combination cannot be used for accurate measurement of fusion or lipid mixing except when bilayers are in the liquid-crystalline state. A more detailed analysis of the properties of NBD-PE as a probe for the T_c and T_H transitions is given elsewhere (Hong et al., 1988).

Lipid mixing assays on SUV (EPE) were also done by the probe dilution method in which both probes were localized in the bilayer of one liposome population at concentrations where resonance energy transfer to Rh occurs. This population was interacted with a 9-fold excess of nonlabeled liposomes. Initial rates of lipid mixing were approximately 10-fold lower for this method, because it is not sensitive to energy transfer between liposome populations upon aggregation, unlike the probe quenching method (Düzgüneş et al., 1987). With the probe dilution method, as with the probe quenching method, we saw no lipid mixing in the T_H region in the absence of Ca^{2+} or Mg^{2+}. The probe dilution method also showed decreases in the slope of initial rates versus temperature at T_H in the presence of Ca^{2+} and Mg^{2+}, similar to the probe quenching method (results not shown). Assays could not be done below T_c because of the marked changes in fluorescence quenching which occurred (see above) making it impossible to calibrate the assay.

SUV of phosphatidylserine can be aggregated by increasing the Na^+ concentration (Day et al., 1980). We have examined the effect of increasing Na^+ concentration on lipid mixing (probe quenching method) for SUV (pH 7.4, 5 mM NaCl) of EPE (Figure 3) and TPE (not shown) at 20 °C. The threshold concentration for aggregation and for lipid mixing mediated by Na^+ ions was 50 mM. Na^+ was able to substitute for Ca^{2+} or Mg^{2+} in promoting aggregation and lipid mixing of PE liposomes, but with a much higher threshold of concentration. This indicated to us that it would be possible to make liposomes at physiological pH in order to look at mixing of contents or contents leakage so long as the ionic strength was kept below the aggregation threshold for the liposomes.

Studies on Leakage of Contents. We examined the rate of calcein leakage from PE liposomes as a function of increasing temperature at different pHs, in the presence or absence of Ca^{2+} or Mg^{2+}, to see if increased rates of leakage were associated with T_H. For SUV, LUV, and MLV (EPE and TPE) in the absence of Ca^{2+} or Mg^{2+}, or at low [Na^+], the rates of leakage of 20 mM calcein remained very low throughout the T_H region. At 50 °C, for example, calcein leakage was 2.9%/min at pH 7.4 and 0.5%/min at pH 9.2 for LUV (EPE) (results not shown). In the presence of Ca^{2+} or Mg^{2+}, the rates of calcein leakage increased substantially for EPE or TPE liposomes, with increases in leakage rate beginning approximately 10 °C below the peak of T_H. For LUV (EPE), this increase in leakage rate began at 35 °C (Figure 4). At 55 °C, calcein leakage from LUV (EPE, 0.1 μm, pH 7.4) in the presence of 1 mM Ca^{2+} averaged 20%/min, and a similar leakage rate was found for LUV (EPE, 0.1 μm, pH 9.2) in the presence of 3.5 mM Ca^{2+}. Divalent cation concentrations of Mg^{2+} (4 mM) which resulted in similar rates of lipid mixing at pH 9.2 resulted in similar rates of leakage (not shown). Increasing the Na^+ concentration to 100 mM in the absence of Ca^{2+} or Mg^{2+} also resulted in increased

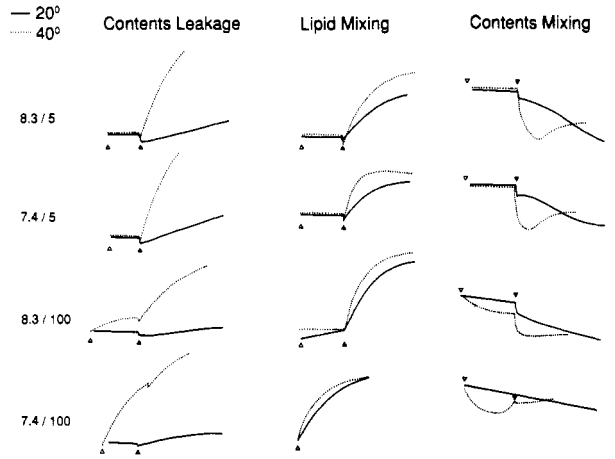

FIGURE 5: Fluorescence changes accompanying lipid mixing (probe dilution method), contents mixing (ANTS/DPX method), and leakage (calcein dequenching method), with changes in pH and ionic strength, and upon addition of 5 mM Ca^{2+}. MLV (0.8 μm) of EPE were made at pH 8.3, 5 mM NaCl, and aliquots of lipid (50 μM) were pipetted into buffer (open arrows) at either pH 8.3, 5 mM NaCl; pH 7.4, 5 mM NaCl; pH 8.4, 100 mM NaCl; or pH 7.4, 100 mM NaCl at the desired temperature. Changes in fluorescence were monitored for 2 or more min, and if no change or slow changes in fluorescence occurred, then 5 mM Ca^{2+} was added to each sample (solid arrows). Solid lines, 20 °C; dotted lines, 40 °C.

calcein leakage beginning at 35 °C for EPE and at 50 °C for TPE at pH 7.4 (results not shown).

Studies on Mixing of Contents. Our results to this point have indicated that suspensions of small or large unilamellar liposomes of EPE or TPE at pH 7.4 or pH 9.2 in the absence of aggregation-inducing cations showed no lipid mixing, no leakage of contents, and no increase in light scattering in the temperature region where pelleted MLV of EPE or TPE at pH 7.4 show H_{II} transitions by DSC. In the presence of Na^+ (higher than 50 mM), Ca^{2+}, or Mg^{2+}, unilamellar liposomes of EPE and TPE show marked changes in light scattering, leakage of entrapped solute (calcein), and alterations in the rate of lipid mixing, near or at their respective T_H. In our next series of experiments, we examined mixing of contents, lipid mixing, leakage of contents, and light scattering in MLV of EPE to see if they behaved differently than unilamellar liposomes. These experiments were all done on a new lot of EPE which did not form liposomes at pH 7.4 at low ionic strength (see Materials and Methods). It was necessary to raise the pH above 7.9 to form stable liposomes, and for this series of experiments, liposomes were made at pH 8.3 (5 mM NaCl). Once liposomes were formed, and extruded, the pH could be dropped to pH 7.4 without causing noticeable instability. Such preparations may contain significant amounts of oligolamellar vesicles. They were stable for at least several hours at 22 °C and several days at 4 °C without any noticeable aggregation as judged by turbidity measurements.

Traces of fluorescent signals for MLV (EPE, extruded through 0.8-μm pore membranes at pH 8.3, 5 mM NaCl) are reproduced in Figure 5 for lipid mixing (probe dilution method), mixing of contents (ANTS/DPX method), and leakage of contents (calcein) at 20 °C, where the liposomes are in the liquid-crystalline state, and at 40 °C, where the liposomes are just below the peak of T_H (Table II). Traces are shown in the absence of Ca^{2+} (beginning at open triangles) and for 2 min following the addition of 5 mM Ca^{2+} (closed triangles). Liposomes were made at pH 8.3 (5 mM NaCl) and were pipetted at a concentration of 50 μM into buffer at the desired pH and ionic strength. At 20 °C, no leakage of contents occurred in the absence of Ca^{2+} in liposomes at pH 8.3 (5 mM NaCl). Neither was there any increase in leakage when the pH was dropped to 7.4 (5 mM NaCl), or when the $[Na^+]$ was raised to 100 mM at pH 8.3, or when the pH was dropped to 7.4 and at the same time as the $[Na^+]$ was raised to 100 mM (Figure 5, solid lines). Addition of 5 mM Ca^{2+} under all of these conditions caused only slow leakage of calcein from the liposomes at 20 °C. On the other hand, increasing $[Na^+]$ to 100 mM at pH 7.4 or at pH 8.3, while not causing much leakage of contents, caused increases in lipid mixing and contents mixing (fusion) at 20 °C (Figure 5, solid lines). This was more marked for liposomes at the lower pH. Addition of 5 mM Ca^{2+} caused additional increases in lipid and contents mixing.

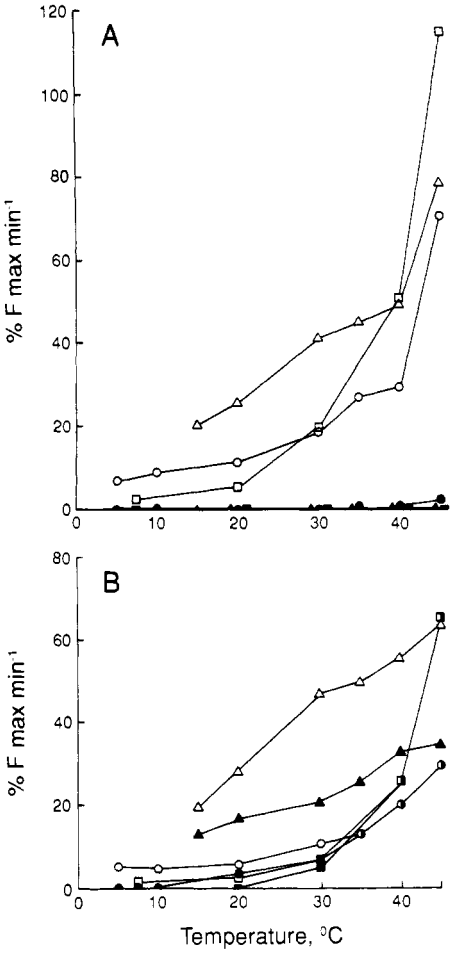

FIGURE 6: Initial rates of leakage (□), mixing of contents (○), and mixing of lipids (△) as a function of temperature for MLV (0.8 μm, EPE) in the absence (closed symbols) or presence (open symbols) of 5 mM Ca^{2+}. (A) pH 7.4, 5 mM NaCl. (B) pH 7.4, 100 mM NaCl.

At 40 °C (Figure 5, dotted lines), mixing of contents, and lipid mixing initiated by increasing Na^+ concentrations, occurred at higher rates than at 20 °C, particularly at pH 7.4 (100 mM NaCl), and this was accompanied by substantial leakage of contents (Figure 5, dotted lines). Addition of 5 mM Ca^{2+} at 40 °C increased the rate of lipid mixing, mixing of contents (fusion), and contents leakage at low or at high ionic strength (Figure 5, dotted lines).

Initial rates of leakage, mixing of lipids, and mixing of contents as a function of temperature are given in Figure 6A,B for MLV (0.8 μm, EPE) in the presence or absence of 5 mM Ca^{2+}. Rates were measured at pHs 7.4 and 8.3 and at low and at high $[Na^+]$. Lipid mixing was analyzed by the probe dilution method which is not sensitive to aggregation.

In the absence of Ca^{2+}, no contents leakage, lipid mixing, or contents mixing was observed for liposomes at low $[Na^+]$

at either pH 7.4 (Figure 6A) or pH 8.3 (not shown). Raising the [Na^+] to 100 mM resulted in a small increase in contents leakage beginning at 40 °C for pH 8.3 liposomes (5–10% maximum, not shown) and a large increase in contents leakage for liposomes at pH 7.4 beginning at 30 °C (Figure 6B). Under all conditions examined, liposomes, irrespective of pH or [Na^+], all showed increased rates of contents leakage in the presence of 5 mM Ca^{2+} as the transition temperature (T_H) was approached. Liposomes in 100 mM NaCl (Figure 6B) were less leaky than liposomes in 5 mM NaCl (Figure 6A) in the presence of 5 mM Ca^{2+}.

In the absence of Ca^{2+}, neither lowering the pH to 7.4 (Figure 6A) nor raising the [Na^+] to 100 mM (not shown) resulted in any significant increase in contents mixing (fusion). However, combining both procedures (Figure 6B) resulted in liposome fusion beginning at 20 °C, as evidenced by high rates of mixing of lipids and contents. No contents leakage occurred at this temperature (Figure 6B). As the temperature increased, so did the initial rates of contents mixing, but leakage of contents also became more pronounced (Figure 5, dotted lines; Figure 6B). The quenching of ANTS fluorescence by DPX is concentration dependent, and as the complex leaks out of liposomes, the fluorescence decrease due to fusion is reversed (Figure 5, dotted lines), and it becomes difficult to measure initial rates of contents mixing in the presence of high rates of contents leakage although it is possible (Nir et al., 1983). In the presence of 5 mM Ca^{2+}, rates of contents mixing were highest for liposomes at low [Na^+] (Figure 6A).

Results for lipid mixing in the absence of Ca^{2+} were qualitatively similar to mixing of contents, with only liposomes at pH 7.4 (100 mM NaCl) showing significant lipid mixing (Figure 6B). In the presence of 5 mM Ca^{2+}, no significant dependence of lipid mixing on pH or ionic strength could be discerned. Rates of lipid mixing were higher than rates of contents mixing.

Discussion

Many of the conclusions which have been drawn about the occurrence of the hexagonal (H_{II}) phase in phosphatidylethanolamine bilayers, and its role in biological processes, have been based on experiments which examined the behavior of closely packed multilayers of phospholipid. Such studies have established a correlation between the occurrence of the hexagonal phase transition, the aggregation of PE bilayers, and their destabilization (Ellens et al., 1986, 1989; Gruner, 1987; Siegel, 1986; Gruner et al., 1988). In the experiments described in this paper, we have examined conditions leading to the formation of the H_{II} phase primarily in dilute suspensions of large or small unilamellar, or multilamellar PE liposomes. From these experiments, we draw the following conclusions, which are graphically represented in a conceptual diagram depicted in Figure 7, where the ionization state of PE is used as one parameter and the temperature as the other.

The diagram is based on phase changes as observed by the calorimeter, and aggregation state as established by optical measurements. Since there is an inherent kinetic parameter in both experimental observations, we do not claim that the phases separated by the lines in the diagram are true equilibrium states. They simply represent different states based on the time scale of the experiments.

First, in our experiments, the H_{II} phase does not appear to occur in dilute unilamellar (or oligolamellar) PE suspensions, at temperatures which thermodynamically favor the H_{II} state in packed multilayers, if the liposomes do not aggregate, i.e., if interbilayer contact does not occur. This is represented by the horizontal (hatched) line a–b in Figure 7, below which the

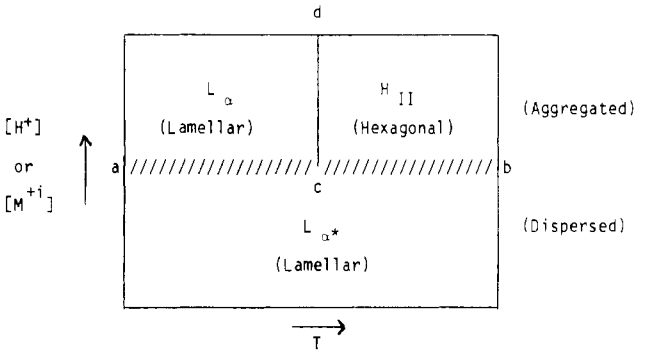

FIGURE 7: Diagrammatic presentation of aggregation, fusion, and phase properties of phosphatidylethanolamines, in relation to pH, metal ion concentration, and temperature. Line a–b: It separates the "aggregated" from the "dispersed" states of PE in aqueous suspensions. This separation is based on observations relating to the kinetics of aggregation and may not reflect a true equilibrium situation. Whether a particular PE preparation is above or below the a–b line depends on the pH and the concentration of monovalent (Na^+) or multivalent (Ca^{2+}, Mg^{2+}) cations. Generally, low pH (below 7.4–8.0) or millimolar concentrations of Ca^{2+} or Mg^{2+} (even at high pH) induce rapid aggregation (above the a–b line). As discussed in the text, the crucial factors affecting the position of the a–b line are the ionization of the PE amino group and the relative ability of metal ions to interact with the head groups at the surface. Additional, possibly kinetic effects are determined by the geometry of the liposomes (MLV vs LUV vs SUV), which show different rates of aggregation. Line c–d: It represents the transition from the lamellar to the hexagonal phase (T_H), and the actual temperature for its occurrence depends on the composition of the acyl chains and to a lesser degree on the nature of the metal ions present.

PE bilayers do not aggregate (within the time limits of our experimental observations, i.e., several hours), and does not show a hexagonal transition (no measurable exothermic reaction within the limits of our calorimetric observations), even at high temperatures. The position of the a–b line is affected both by pH and by the concentration as well as valency of metal ions. It is also affected by the geometry of the vesicles (MLV vs LUV and SUV) and the time allowed for observation. This line therefore represents a threshold between aggregated and nonaggregated states only within the confines of the experimental conditions employed. Aggregation is prevented when the liposomes carry sufficient net negative charge, and the pH at which this occurs depends primarily on the pK_a of the amine group which has been estimated to be approximately 9.5 (Tsui et al., 1986). From our experiments with egg PE, inhibition of aggregation is observed in the region of pH 7.4–8.0, when only a small percentage of amino groups would be expected to be deprotonated. This results in a small but measurable net negative surface charge. Liposomes maintained above this pH range do not aggregate (within several hours at room temperature or days at 4 °C). However, rapid aggregation can be induced by a number of manipulations. Lowering the pH (addition of H^+ ions) and thus reducing the net negative charge of the PE liposomes will induce aggregation (above the a–b line in Figure 7) and lead to formation of the H_{II} phase when the temperature is raised through the H_{II} transition temperature (vertical c–d line in Figure 7). At pHs slightly higher than those needed to induce aggregation by H^+ ions, addition of Na^+ ions will induce aggregation, and the liposomes will undergo the H_{II} transition. At the pH where aggregation can be induced by Na^+ ions, packing of the liposomes by centrifugation seems to induce sufficient bilayer contact for formation of the H_{II} phase at the H_{II} transition temperature, when the radius of curvature exceeds a critical value of >100 nm. At still higher pH, where the membranes carry more negative charge, centrifugation or

addition of Na^+ ions is insufficient to cause membrane aggregation and lead to formation of the H_{II} phase, but membrane aggregation and H_{II}-phase formation can be brought about by divalent cations such as Ca^{2+} and Mg^{2+}. As the pH increases further, higher concentrations of Ca^{2+} and Mg^{2+} are necessary for formation of H_{II}-phase lipid.

Second, formation of the H_{II} phase is associated with a high rate of leakage of entrapped contents from PE liposomes. Fusion of PE liposomes will occur under conditions leading to formation of the H_{II} phase as evidenced by assays measuring mixing of contents or lipids, and by changes in light scattering, but this fusion is accompanied by rapid loss of liposome contents.

Third, PE liposomes, under nonaggregating conditions, will exist in the bilayer (L_α) state at temperatures considerably higher than those leading to formation of H_{II} lipid under aggregating conditions. These liposomes are stable within the time of our observations and do not release their contents at temperatures well above the H_{II} transition temperature. When PE liposomes are induced to aggregate below the H_{II} transition temperature, then fusion occurs without significant loss of entrapped contents. Fusion can be induced in the L_α or the H_{II} state by any metal ion or H^+ ions so long as the liposomes can be induced to aggregate.

Finally, there does not appear to be any exclusive correlation between fusion and formation of the hexagonal phase. Nonleaky fusion occurs between PE liposomes at temperatures of 20–25 °C which are below the temperatures which thermodynamically favor the formation of the H_{II} phase (T_H). There is, however, a strong correlation between formation of the H_{II} phase and leakage of contents.

We can therefore identify three states[2] for the PE membranes above the temperature for the solid to fluid transition, T_c (Figure 7): first, a state (L_α, fluid lamellar) in which there is no aggregation, no lipid or contents mixing, no liposome contents leakage, and no observable H_{II} transition, even at H_{II}-permissive temperatures (above the a–b line, Figure 7); second, a state[3] in which aggregation induced by, for example, increasing $[H^+]$, $[Na^+]$, $[Ca^{2+}]$, or $[Mg^{2+}]$ leads to formation of the H_{II} phase at high temperatures, accompanied by lipid and contents mixing, as well as substantial contents leakage (above the a–b line, and to the right of the c–d line, Figure 7); third, an aggregated state induced by the above manipulations, at temperatures where the membrane is in the L_α state, which leads to contents mixing (fusion) and lipid mixing but with considerably reduced leakage of contents (above the a–b line, and to the left of the c–d line of Figure 7).

Multilamellar (MLV) PE suspensions show a higher tendency for both aggregation and the formation of hexagonal transitions at higher temperatures (Gruner et al., 1988) when compared to LUV or SUV at similar conditions of pH and ionic strength. Whether this relates to curvature effects and differences in the kinetics of aggregation or to other reasons is not clear at present. It is quite likely that SUV and LUV may not be at true equilibrium and that MLV may be closer to that state. However, for understanding cell membrane phenomena, the interactions between SUV or LUV may be equally or more relevant than MLV. In any case, it is important to use similar types of vesicles when comparing structural characteristics with fusion, although in most cases in the past, the structure was studied with MLV, while fusion was studied with LUV (Ellens et al., 1989).

The results obtained by high-sensitivity DSC demonstrate that larger unilamellar liposomes of EPE in suspension can undergo the H_{II} transition at pH 7.4, at low salt concentrations. Under the same conditions, but with a reduced liposome size, the H_{II} transition is diminished and becomes very broad, as shown in Figure 1C. These transitions have low enthalpies compared to those obtained with pelleted liposomes. Since larger LUV preparations tend to have some populations of liposomes with multilamellar or oligolamellar structure, it is possible that the H_{II} transitions with small enthalpies observed in Figure 1B,C are associated with those multilamellar populations. Repetitive extrusion sequentially through smaller pore size filters not only reduces the average size but also reduces the number of lamellae (Mayer et al., 1986; Jousma et al., 1987) which could lead to broadening and disappearance of the T_H, due to the lower cooperativity and other constraints associated with smaller liposomes. The size or multilamellarity requirement for undergoing the H_{II} transition cannot be resolved at this time. The fact remains, however, that small PE liposomes in suspension do not show evidence for a H_{II} transition either by calorimetry or through lack of aggregation and release of contents within the limits of our experimental observations.

Siegel (1984) has made thermodynamic calculations which demonstrate that inverted micellar intermediates (IMI, structures which form between opposed bilayers) are likely intermediates in lamellar to H_{II}-phase transitions. These structures have also been implicated as intermediates in the fusion process (Verkleij et al., 1980; Verkleij, 1984). Hope et al. (1983) have reported from freeze–fracture studies that vesicles consisting of PE and negatively charged phospholipids fuse prior to H_{II}-phase formation and that this fusion is accompanied by the appearance of IMI. However, other studies employing fast-freezing techniques (Bearer et al., 1982; Verkleij, 1984) suggests that fusion precedes the appearance of IMI. The difficulty of imaging IMI in fast-freezing experiments due to their short half-lives has been pointed out by Siegel (1984). From the present experiments, we cannot draw conclusions as to whether or not lipidic intermembrane particles represent a fusion intermediate. The final equilibrium phase (H_{II}), which does not appear to be involved in fusion of PE liposomes, cannot be equated with the occurrence of IMI which, if they are functioning as fusion intermediates, will be unstable and short-lived.

Fusion between membranes involves two distinct sequential processes: first, aggregation of two or more vesicles with close apposition of their bilayers: second, a destabilization process leading to merging of the bilayers and mixing of contents of each vesicle (Nir et al., 1983). In the case of phosphatidylserine liposomes, these two processes can be shown to be independent (Düzgüneş & Papahadjopoulos, 1983). With Mg^{2+} or high concentrations of monovalent cations, the vesicles can be made to aggregate reversibly (Day et al., 1980; Nir et al., 1981; Wilschut et al., 1981), but they will not fuse unless Ca^{2+} is present to initiate membrane destabilization, possibly through its capability to induce dehydration and phase separation in the phosphatidylserine bilayers (Portis et al., 1979; Bentz et al., 1983; Bentz & Ellens, 1988).

In the case of PE liposomes, the two processes do not appear to be separable. Conditions which lead to membrane aggregation are accompanied by mixing of lipid and liposome

[2] We use the term "state" in the context of our experimental conditions which may not be at true equilibrium. The time scale of our experiments was of the order of minutes to hours, which is the time of interest for fusion, aggregation, and leakage experiments.

[3] Small differences were observed within this particular state (Table I) depending on whether the counterion was a proton, Na^+, Ca^{2+}, or Mg^{2+}, probably reflecting differences in complexation at the head groups.

contents. PE can be considered as forming a metastable bilayer which, under conditions of close intermembrane contact, has a low energetic threshold for rearrangement to nonbilayer forms like IMI which lead to fusion events. Liposomes of PE can be thought of as existing in a state of metastable equilibrium where small changes in pH, ionic strength, or divalent cation concentration can lead to substantial changes in aggregation and phase state of the membranes. This behavior of PE may be related to its ability to form intermolecular hydrogen bonds between the negatively charged phosphate and the protonated amine at lower pH (Papahadjopoulos, 1968; Boggs, 1980). PE, under conditions of pH where intermolecular hydrogen bonding can occur, hydrates very poorly. Raising the pH, which results in deprotonation of the amine and presumably disruption of intermolecular hydrogen bonds, leads to an increase of the degree of hydration (possibly involving hydrogen bonding between water and individual phosphate and amino groups) and the formation of stable lamellar structures. Lowering the pH, leading to intermolecular hydrogen bond formation, or other manipulations causing dehydration encourage interbilayer contact which in turn can lead to molecular rearrangements such as fusion and/or formation of H_{II}-phase lipid.

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Registry No. H^+, 12408-02-5; Na, 7440-23-5; Ca, 7440-70-2; Mg, 7439-95-4.

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Effect of Ionic Strength and Cationic DNA Affinity Binders on the DNA Sequence Selective Alkylation of Guanine N7-Positions by Nitrogen Mustards

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ABSTRACT: Large variations in alkylation intensities exist among guanines in a DNA sequence following treatment with chemotherapeutic alkylating agents such as nitrogen mustards, and the substituent attached to the reactive group can impose a distinct sequence preference for reaction. In order to understand further the structural and electrostatic factors which determine the sequence selectivity of alkylation reactions, the effect of increased ionic strength, the intercalator ethidium bromide, AT-specific minor groove binders distamycin A and netropsin, and the polyamine spermine on guanine N7-alkylation by L-phenylalanine mustard (L-Pam), uracil mustard (UM), and quinacrine mustard (QM) was investigated with a modification of the guanine-specific chemical cleavage technique for DNA sequencing. For L-Pam and UM, increased ionic strength and the cationic DNA affinity binders dose dependently inhibited the alkylation. QM alkylation was less inhibited by salt (100 mM NaCl), ethidium (10 μM), and spermine (10 μM). Distamycin A and netropsin (100 μM) gave an enhancement of overall QM alkylation. More interestingly, the pattern of guanine N7-alkylation was qualitatively altered by ethidium bromide, distamycin A, and netropsin. The result differed with both the nitrogen mustard (L-Pam < UM < QM) and the cationic agent used. The effect, which resulted in both enhancement and suppression of alkylation sites, was most striking in the case of netropsin and distamycin A, which differed from each other. DNA footprinting indicated that selective binding to AT sequences in the minor groove of DNA can have long-range effects on the alkylation pattern of DNA in the major groove.

Bis(2-chloroethyl)methylamine (mechlorethamine, nitrogen mustard) was the first clinically effective anticancer agent (Gilman & Philips, 1946), and derivatives such as L-phenylalanine mustard (melphalan, L-Pam),[1] cyclophosphamide, and chlorambucil are still among the most useful clinical agents (Haskel, 1985). Covalent binding may occur at many nucleophilic sites within nucleic acids and proteins, but DNA is probably the most important target with reaction predominantly at the N7-position of guanine (Lawley, 1966; Singer, 1975). The requirement for antitumor activity of two alkylating groups within the mustard molecule suggests that the activity arises from the formation of cross-links between macromolecular sites (Lawley, 1966; Kohn et al., 1966; Kohn, 1980). DNA interstrand cross-links and DNA–protein cross-links have been observed in intact cells (Ewig & Kohn, 1977), and evidence for intrastrand cross-links has been obtained indirectly (Chun et al., 1969).

A modification of the Maxam and Gilbert (1980) guanine-specific chemical cleavage technique for DNA sequencing has allowed a direct examination of the guanine N7 reaction of alkylating agents at the individual base level in purified DNA (Mattes et al., 1986a). Large variations in alkylation intensities existed among guanines in a DNA sequence following treatment with nitrogen mustards (Mattes et al., 1986b; Kohn et al., 1987), *N*-alkyl-*N*-nitrosoureas (Hartley et al., 1986; Wardeman & Gold, 1988), and triazenes (Hartley et al., 1988b). The most striking finding was that most agents reacted preferentially in runs of guanines, the degree of preference being much greater than would be expected from the number of guanines alone. This correlated well with the molecular electrostatic potential at the guanine N7-position imposed by the nearest-neighbor base pairs (Pullman & Pullman, 1981) and suggests that the specific biological effects of such compounds may depend on preferential reaction at GC-rich genomic locations (Mattes et al., 1988). In addition, some nitrogen mustards (in particular uracil and quinacrine mustards, Figure 1) showed distinctly different reaction patterns from other mustards, indicating that the substituent attached to the reactive group could impose a distinct sequence preference for reaction (Mattes et al., 1986), and models to explain this have been proposed (Kohn et al., 1987).

In order to understand further the structural and electrostatic factors which determine the sequence selectivity of alkylation reactions, the present study was undertaken to investigate the effect of cationic DNA binders on guanine N7-

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[1] Abbreviations: L-Pam, L-phenylalanine mustard (melphalan); UM, uracil mustard; QM, quinacrine mustard; MPE, methidiumpropyl-EDTA; MNU, *N*-methyl-*N*-nitrosourea; EDTA, ethylenediaminetetraacetic acid.

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