Hydrophobic Lipid Additives Affect Membrane Stability and Phase Behavior of *N*-Monomethyldioleoylphosphatidylethanolamine†

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ABSTRACT: The rate of formation of high-curvature intermediates or disordered cubic phases in *N*-methyldioleoylphosphatidylethanolamine (*N*-methyl-DOPE) dispersions with or without additives was studied by ^{31}P NMR spectroscopy. In *N*-methyl-DOPE dispersions, both the L_α liquid-crystalline phase and the hexagonal H_{II} phase convert into phases of high curvature giving rise to isotropic ^{31}P NMR resonances. Addition of the bilayer destabilizers 1,2-diolein, 1,3-diolein, or eicosane lowers the threshold temperature of the isotropic phase. The isotropic threshold temperature is strongly correlated with the L_α–H_{II} phase transition temperature (T_H). The addition of hexagonal phase promoters does not change the rate of formation of the isotropic phase at a temperature shifted by a fixed amount below T_H. However, the formation of "isotropic" phases from the additive-stabilized hexagonal phase is slow compared to that observed in pure *N*-methyl-DOPE lipid dispersions. Membrane leakage and fusion are promoted by the dioleins and well as by eicosane, but changes in the rates of these processes do not correlate well with the extent of formation of isotropic phases. All three additives have similar effects on phase behavior and on vesicle leakage and fusion. These similarities occur despite the fact that eicosane is believed to partition differently into the membrane than diolein. In addition to the general similarities in the effects of the two diolein isomers, 1,2-diolein is somewhat more potent in promoting the hexagonal phase and in increasing rates of leakage and fusion than is 1,3-diolein.

The synthetic lipid *N*-methyl-DOPE[1] is capable of forming cubic phases with symmetry point groups of *Im3m* and *Pn3m*, as determined by X-ray diffraction studies (Gruner et al., 1988; Siegel & Banschbach, 1990). The rate of formation of the metastable cubic arrays from the L_α bilayer phase is slow, but it is rapid when cooling down from the hexagonal phase (H_{II}). The addition of 1,2-diacylglycerol lowers the temperature at which the formation of isotropic phases occurs as determined by ^{31}P NMR spectroscopy (Ellens et al., 1989; Siegel et al., 1989a). The formation of these isotropic phases, which are indicative of high curvature, are believed to be involved in fusion processes. Because of the facility with which *N*-methyl-DOPE forms isotropic phases, it provides a suitable model system to evaluate the role of this structure in the functional properties of membranes. The appearance of intermediates prior to the formation of the cubic phases may accelerate liposome fusion. The temperature dependence of the formation of the isotropic phase is sensitive to the presence of certain additives in the membrane. We investigated the difference between the addition of 1,2-diolein, 1,3-diolein, or eicosane (a C-20 saturated straight-chain alkane) on the rate of formation of regions of high curvature and on membrane fusion and leakage. Two of the factors that determine the relative stability of bilayer and nonbilayer phases are the intrinsic radius of curvature of each monolayer of a bilayer and the relief of hydrocarbon packing constraints in the H_{II} phase (Gruner, 1985; Siegel et al., 1989b). While no additive to the membrane is likely to affect only one of these two factors, alkanes which can partition into the voids between the hexagonal phase cylinders would primarily relieve the hydrocarbon packing constraints in the H_{II} phase (Gruner, 1985). This may be less true for the long alkane, eicosane, used in the present work, but even with eicosane the relief of hydrocarbon packing constraints should be complete. Eicosane was chosen to avoid volatility of the alkane. In comparison, diacylglycerols partition into the membrane with their hydroxyl groups positioned at the membrane surface (Hamilton et al., 1991a,b). As a consequence, they should have less effect on hydrocarbon packing constraints in the H_{II} phase, but, because of the high ratio of hydrophobic to hydrophilic volumes, they should alter the intrinsic radius of the curvature of the lipid–water interface. We wished to determine how these two types of "bilayer destabilizers" affect membrane properties such as leakage and fusion.

In addition to affecting the rates of membrane leakage and fusion, changes in the physical properties of membranes will also alter the activity of some membrane-bound enzymes. Protein kinase C (PKC) is an important enzyme for the regulation of cell function. The activity of the enzyme is dependent on phosphatidylserine and Ca^{2+}, but at low calcium concentrations it is markedly activated by 1,2-diacylglycerols (Nishizuka, 1986). Diacylglycerols are believed to serve an important physiological role in the regulation of the activity of this enzyme. The activation of PKC by diacylglycerols is stereospecific, with neither 1,3-diolein or 2,3-diolein generally

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[1] Abbreviations: *N*-methyl-DOPE; *N*-methyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; $L\alpha$, liquid crystalline lamellar phase; H_{II}, inverted hexagonal phase; Iso, phase which gives rise to a narrow-line NMR spectrum; T_H, L_α–H_{II} phase transition temperature; T_I, onset temperature of L_α–Iso transition; LUV, large unilamellar vesicle; DPX, *p*-xylylenebis(pyridinium bromide); ANTS, aminonaphthalene-3,6,8-trisulfonic acid; NBD-PE, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PKC, protein kinase C; RET, resonance energy transfer fluorescence assay of lipid intermixing.

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being able to substitute for 1,2-diolein (Boni & Rando, 1985; Molleyres & Rando, 1988). We wished to determine whether the effects of 1,2- vs 1,3-diolein on the membrane physical properties could contribute to making the former lipid a more potent activator of PKC. Recent reports indicate that these two isomers have similar effects in lowering T_H using dielaidoylphosphatidylethanolamine. The 1,2-isomer is slightly more potent than the 1,3-diolein (Micol et al., 1990). However, when the effect of the diolein isomers on the calcium-induced fusion of phosphatidylserine–phosphatidylcholine (1:1) vesicles was measured by the mixing of aqueous contents, the 1,2-diolein was much more effective in promoting fusion (Gômez-Fernândez et al., 1990). We wished to determine how leakage and fusion were affected in a vesicle system which had a marked propensity to undergo transitions to nonbilayer phases. The effects of the dioleins on membrane phase behavior and on leakage and fusion are indicative of how these substances alter the physical properties of membranes. If the behavior of the two isomers is different, then we can determine if this difference could also be related to the observed differences in the potency of the two isomers in activating PKC.

Materials and Methods

Chemicals. All lipids were commercially available and used without further purification: *N*-methyl-DOPE (Avanti Polar Lipids Inc.), 1,2-diolein (Serdary Research Laboratories), 1,3-diolein (Sigma), and eicosane (Nu-Check Prep). The lipid preparations were shown to be pure by the criterion of thin layer chromatography. Under the conditions used, the 1,2- and 1,3-isomers of diolein could be separated. ANTS and DPX are from Molecular Probes (Junction City, OR).

^{31}P NMR Spectroscopy. The lipids were dissolved together in chloroform/methanol, dried under vacuum, and resuspended in 20 mM HEPES buffer, 150 mM NaCl, and 1 mM EDTA, pH 7.4. The lipid to buffer weight ratio was 1:1 for all ^{31}P NMR experiments. The lipid dispersions were vigorously vortexed and freeze–thawed five times using liquid nitrogen. All NMR spectra were recorded on a Bruker WM-250 NMR spectrometer at 101.23 MHz using a single-pulse experiment and broad-band proton decoupling. The initial delay was kept short (5 μs) to avoid line shape distortions due to angular dependent spin–spin relaxation times (Sternin, 1988). The temperature was controlled to an accuracy of ± 1 °C with a Bruker variable temperature unit (BVT-100) using liquid nitrogen for lower temperatures and nitrogen gas flow for temperatures above 45 °C. All lipid dispersions were frozen in liquid nitrogen for 15 min prior to the NMR experiments. They were thawed at room temperature and allowed to equilibrate for 1 h at room temperature. Typically, 1024 scans were accumulated with a relaxation delay of 3 s. The time allowed for temperature equilibration after a 5 °C rise in temperature was 30 min. The time-dependent studies were performed by allowing the temperature to rise from an equilibrated state of the lipid dispersion in which no isotropic resonance was observed to a given temperature and appropriate equilibration (15–30 min). Fast temperature equilibration was obtained by using small volumes (100 mg of lipid in 100 μL of buffer). The sample was contained in 5-mm diameter thin-walled NMR tubes. The high lipid concentration also aided the rapid formation of nonbilayer phases. After temperature equilibration, either 256 or 512 scans were recorded using a repetition delay of 3s. The number of experiments varied between 10 and 60 depending on the rate of formation of the isotropic ^{31}P NMR signal. Other spectral parameters were sweep width, 50 kHz; time domain, 4096 points; acquisition time, 41 ms; and pulse width (90° pulse), 76 μs. The half-time was calculated for the formation rates of the isotropic phases in each experiment. Exponential line-broadening of 50–150 Hz was applied prior to Fourier transformation. The intensities of the spectral components were determined after deconvolution, which was facilitated by the sharp isotropic resonance. The initial formation rate of the isotropic ^{31}P NMR phase was graphically determined from the plot of the relative isotropic signal intensity vs time.

Differential Scanning Calorimetry. DSC studies were performed on a Microcal MC2 calorimeter, using a liquid sample holder (1.24 mL) and a scan rate of 45 °C/h. Both the reference buffer and the sample were degassed before the DSC run. The lipid dispersions were frozen in liquid nitrogen prior to degassing, and the typical sample concentration was 10 mg/mL in 20 mM HEPES, 150 mM NaCl, and 1 mM EDTA, pH 7.4.

Leakage Studies. Desired amounts of *N*-methyl-DOPE and additives were dissolved in chloroform/methanol and dried under vacuum. The lipid films were resuspended in 1 mL of 12.5 mM ANTS, 45 mM DPX, 22.5 mM NaCl, and 10 mM glycine (pH 9.5), subjected to five freeze-thaw cycles, and extruded 10 times through an 0.1-μm polycarbonate filters (Lipex Biomembranes, Vancouver BC, Canada). The ANTS/DPX-containing lipsomes were eluted over a Sephadex G-75 column using a 10 mM glycine buffer (pH 9.5, 100 mM NaCl, 0.1 mM EDTA). The leakage experiments were performed using 100 nmol of phospholipid in the form of LUV's in a 700-μL total volume. The emission intensity was measured at 530 nm using a excitation wavelength of 360 nm on a Perkin-Elmer MPF-44 spectrofluorometer. At the initial fluorescence, $F(0)$, no leakage had occurred. After injection of 15 μL of 0.4 mM $MgCl_2$ and acidification to pH 7.4, the fluorescence $F(t)$ was measured with time. The 100% leakage $F(100)$ was obtained after addition of 10 μL of 10% Triton X-100. The amount of leakage at any given time is given by: % leakage = $[F(t) - F(0)]/[F(100) - F(0)]$.

Contents-Mixing Assay. The lipid films were hydrated with buffer containing either 25 mM ANTS, 45 mM NaCl, and 10 mM glycine (pH 9.5) or 90 mM DPX and 10 mM glycine (pH 9.5) (Ellens et al., 1989). Equal amounts (50 nmol) of LUV's containing ANTS or DPX were mixed in glycine buffer (pH 9.5) to a total volume of 700 μL. After injection of 15 μL of 0.4 M $MgCl_2$ and acidification to pH 7.4, the maximum emission at 530 nm was measured as a function of time on a Perkin-Elmer MPF-44 spectrofluorometer (λ_{ex} = 360 nm).

Lipid-Intermixing (RET) Assay. Processes such as membrane fusion which lead to the intermixing of lipid from different vesicles can be monitored by an assay based on fluorescence resonance energy transfer (RET) between labeled lipid molecules (Struck et al., 1981). Lipid films of *N*-methyl-DOPE either with (labeled) or without (unlabeled) 1 mol % *N*-NBD-PE and 1 mol % *N*-Rh-PE were prepared as described above, but without passage through Sephadex. To 700 μL of 10 mM glycine buffer (pH 9.5), 10 nmol of labeled LUV's and 50 nmol of unlabeled LUV's were added. After injection of 15 μL 0.4 M $MgCl_2$ and acidification to pH 7.4, the fluorescence emission intensity at 530 nm was measured with time (λ_{ex} = 450 nm).

Results

^{31}P NMR Spectroscopy. Lipid dispersions of *N*-methyl-DOPE at 25 °C show ^{31}P NMR powder patterns indicative of a liquid-crystalline bilayer arrangement with axially symmetric motions. The formation of an isotropic signal was not observed upon heating until a temperature of 55 °C, which is much higher than previously reported for this lipid using

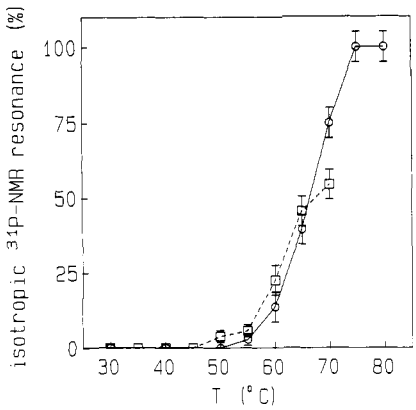

FIGURE 1: Relative amount of the isotropic ^{31}P NMR phase with respect to the total signal intensity in *N*-methyl-DOPE dispersions at varying temperatures. The spectral intensities were determined after deconvolution. The lipid suspension (100 mg/100 μL) was gradually heated from room temperature (see Materials and Methods for details). Fresh and properly hydrated *N*-methyl-DOPE dispersion (○); *N*-methyl-DOPE dispersion in 5 mM Mg^{2+} buffer (□).

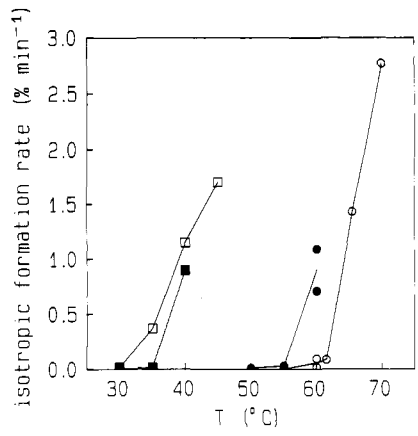

FIGURE 2: Initial formation rate of the isotropic ^{31}P NMR phase at varying temperatures for *N*-methyl-DOPE dispersions (○); *N*-methyl-DOPE + 5 mM Mg^{2+} (●); *N*-methyl-DOPE + 2 mol % 1,2-diolein (□); and *N*-methyl-DOPE + 2 mol % 1,3-diolein (■).

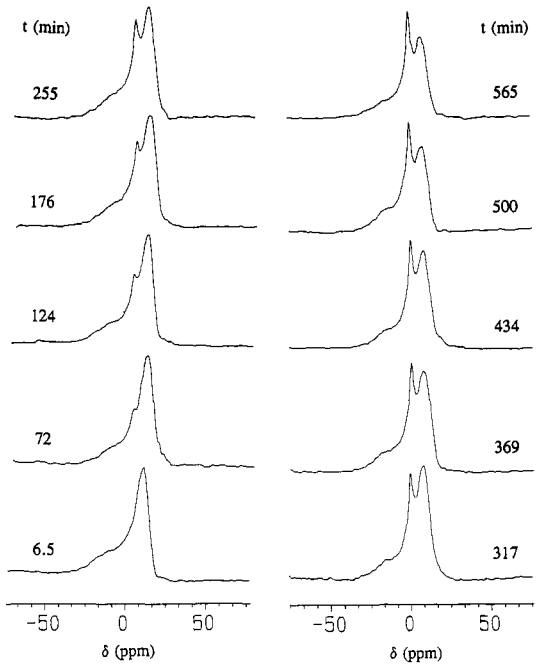

FIGURE 3: Time-dependent ^{31}P NMR spectra of *N*-methyl-DOPE in 5 mM Mg^{2+} buffer at 55 °C. The lipid concentration was 50 wt % and the NMR parameters are given under Materials and Methods.

different protocols (Ellens et al., 1989; Siegel et al., 1989a; Gagne et al., 1985). The amount of isotropic phase increases with increasing temperatures although the total integrated area of the ^{31}P NMR spectrum remains constant. The ^{31}P NMR spectra of pure *N*-methyl-DOPE show complete conversion into an isotropic resonance at higher temperature (Figure 1). The loss of the ^{31}P NMR bilayer powder pattern is not reversible, and upon cooling from the hexagonal–isotropic phase mixture a completely isotropic signal is observed at room temperature. The only method by which we could reset the system was by lowering the temperature well below the gel–liquid-crystalline phase transition temperature, in agreement with previous observations (Gruner et al., 1988).

The amount of isotropic signal observed in the ^{31}P NMR spectra is often time-dependent. From X-ray diffraction (Gruner et al., 1988) as well as from differential scanning calorimetry (Siegel & Banschbach, 1990), it is known that the "cubic" phase is formed slowly. In this study, we have investigated the formation of the isotropic ^{31}P NMR phase over time. The formation rate of the isotropic phase was measured in *N*-methyl-DOPE lamellar dispersions at five temperatures. The isotropic phase is formed from both the L_α phase ($T < 66$ °C) as well as from the H_{II} phase ($T > 70$ °C). Below 60 °C the formation rate is extremely slow (<0.01% min^{-1}). After 24 h, the amount of isotropic signal intensity was 2% and 14% for temperatures of 55 and 60 °C, respectively. At 61–62 °C, the formation rate is 0.09% min^{-1}, and the amount of isotropic ^{31}P NMR signal increases until it reaches approximately 70–75% of isotropic phase. We determined by DSC using a solid cell and slow scan rates (2.3 °C/h) that the midpoint of the L_α–Iso transition occurred at 60.8 °C. At 66 °C, the rate of formation of the isotropic phase out of a liquid-crystalline bilayer has increased significantly to a rate of 1.4% min^{-1}. The hexagonal H_{II} phase is converted into the isotropic phase at 70 °C at a fast rate of 2.8% min^{-1}. The initial formation rates of the isotropic phase are strongly dependent on temperature (Figure 2). The addition of 5 mM Mg^{2+}, needed to induce vesicle–vesicle fusion, lowers the temperature at which the isotropic phase was first observed by ~4 °C (Figure 2). Addition of Mg^{2+} also lowered T_H by ~4 °C. Time-dependent spectra of *N*-methyl-DOPE in 5 mM Mg^{2+} buffer at 55 °C are shown in Figure 3.

We have studied the effect of three additives on the formation of the isotropic and hexagonal phases in *N*-methyl-DOPE dispersion. The addition of all three substances results in a lowering of T_I and T_H as indicated by changes in the ^{31}P NMR spectra (Figure 4). However, 1,2-diolein is the most potent, and the isotropic signal is initially observed at a temperature of 30 °C. The isotropic phase always appears at lower temperatures than the hexagonal phase, but the phase transitions are broad in the presence of additives, and three-component ^{31}P NMR spectra were observed over a wide tem-

Table I: Onset Temperature of the Lamellar to Isotropic Phase Transition (T_I) and the Lamellar–Hexagonal Phase Transition Temperature (T_H) for *N*-Methyl-DOPE Lipid Dispersions[a]

liposome composition	T_I (°C)	T_H (°C)
N-methyl-DOPE	55	67
"old" dispersion (1–2 weeks)	42	57
N-methyl-DOPE + 5 mM $MgCl_2$	50	62
N-methyl-DOPE + 2% 1,2-diolein	30	43
N-methyl-DOPE + 2% 1,3-diolein	35	47
N-methyl-DOPE + 2% eicosane	34	44

[a] Both T_I and T_H were estimated from the temperature-dependent ^{31}P NMR spectra. The lipid concentration was 50 wt %. Experimental parameters for NMR acquisition are given under Materials and Methods.

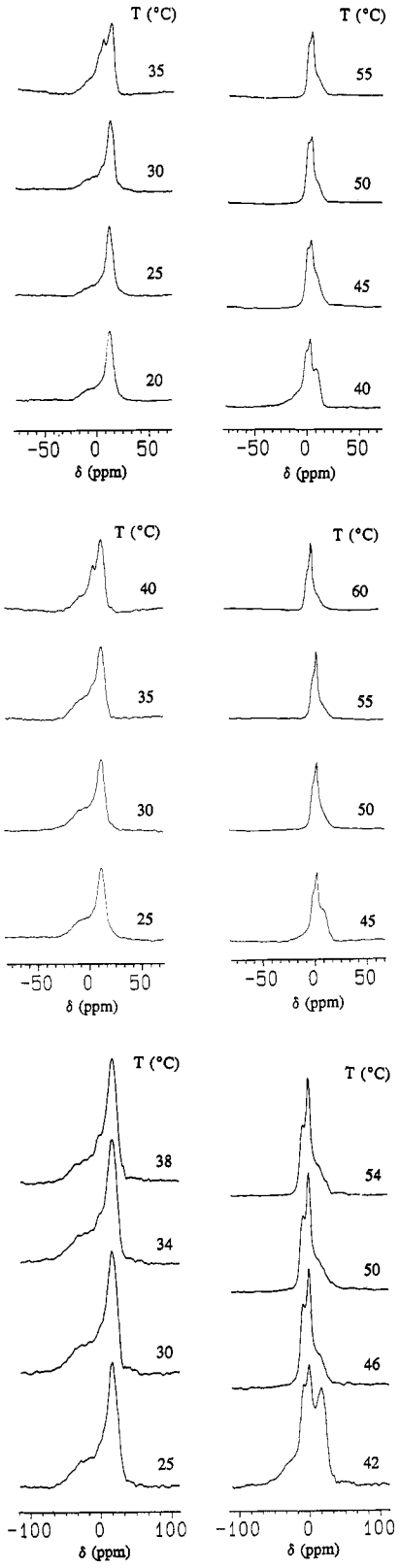

FIGURE 4: Temperature-dependent ^{31}P NMR spectra of *N*-methyl-DOPE with 2 mol % additive, (a, top) 1,2-diolein, (b, middle) 1,3-diolein, and, (c, bottom) eicosane. Samples were incubated for 30 min after each 5 °C increase in temperature (see Materials and Methods).

perature interval. The temperature at which the isotropic peak first appears, T_I, shows a linear correlation (correlation coefficient = 0.98) with T_H (Table I). The formation rate of the isotropic phase was time-dependent and was measured for *N*-methyl-DOPE in the presence and absence of either 2% 1,2-diolein or 1,3-diolein at various temperatures (Figure 2).

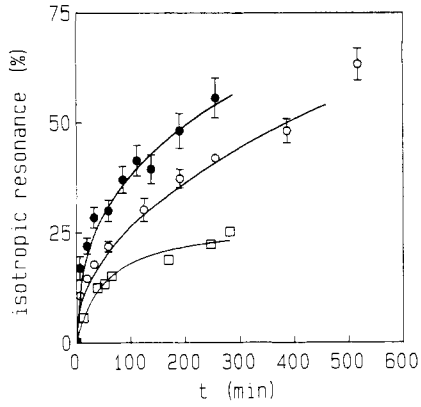

FIGURE 5: Time-dependent increase of the isotropic ^{31}P NMR phase in 2% 1,2-diolein containing *N*-methyl-DOPE liposomes at 35 (□), 40 (○), and 45 °C (●). The isotropic signal intensity was determined after spectral deconvolution. The initial formation rate of the isotropic phase was determined graphically from these plots.

In contrast to the pure lipid, the ^{31}P NMR spectra of *N*-methyl-DOPE with additives do not show complete conversion into an isotropic phase at any temperature. The maximum amount of isotropic phase measured in *N*-methyl-DOPE with additives after 30 min of incubation was between 14% and 19% (14% for 1,2-diolein, 17% for eicosane, and 19% for 1,3-diolein) of the total spectral intensity and remained unchanged above the L_α–H_{II} phase transition. However, in the time-dependent NMR experiments of *N*-methyl-DOPE liposomes containing 2 mol % of 1,2-diolein, we observed up to 60% of isotropic phases at 45 °C (Figure 5).

During the long time-dependent NMR experiments, acyl chain migration does occur in lipid dispersions containing 1,2-diolein (Hamilton et al., 1991a,b; Kodali et al., 1990). We also observed that at high temperatures up to 30% of the 1,2-isomer was converted into 1,3-diolein as estimated from the spot intensities on a TLC plate (Silica, hexane/ether, 1:1). This will reduce the potent effect of the 1,2-isomer on the L_α–H_{II} phase transition temperature as well as the temperature at which the isotropic phase was first observed.

We have studied the differences between the isomers 1,2- and 1,3-diolein on the L_α–H_{II} transition of dielaidoylphosphatidylethanolamine. The bilayer destabilizing potency of 1,2-diolein is reflected in the large negative slope of a plot of T_H vs mole fraction diolein (−1020 ± 20 °C/mole fraction additive) compared to 1,3-diolein (−630 ± 30 °C/mole fraction additive). We also studied the effect of both additives on the phase behavior of *N*-methyl-DOPE but used a lower mole fraction of additives. Addition of 1.0 mol % of additive resulted in a decrease in the L_α–H_{II} phase transition temperature from 66.7 °C for pure *N*-methyl-DOPE to 57.8 °C with 1,2-diolein and to 58.6 °C (61.9 °C for 0.5 mol %) with 1,3-diolein. The DSC results again indicate the irreversibility upon cooling, and the reheat from 20 °C upward shows only the broad isotropic–hexagonal phase transition. The formation of the isotropic phase could not be observed in the liquid sample cell of the Microcal MC-2 calorimeter, using a scan rate of 45 °C/h. Only the use of solid cell holder at high lipid concentrations and slow scan rates (<10 °C/h) shows the endothermic transition of the L_α–isotropic phase transition. (For *N*-methyl-DOPE, T_I = 60.8 °C with a 2.3 °C/h scan rate).

Figure 6 shows the initial rate of leakage at different temperatures. A sharp increase in the initial rate of leakage is observed for temperatures above T_H. Once again, 1,2-diolein shows the largest effect. Eicosane and 1,3-diolein have similar effects on the leakage rate. The pure lipid, *N*-methyl-DOPE, shows the least leakage.

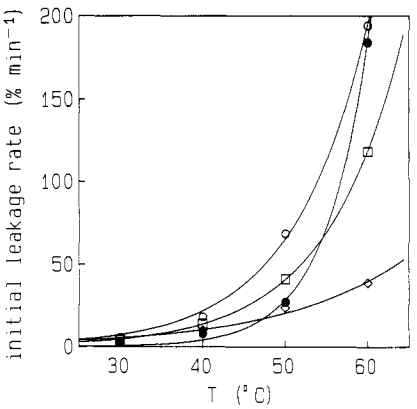

FIGURE 6: Initial leakage rate at different temperatures for various lipid dispersions: *N*-methyl-DOPE lipid dispersion (◇), *N*-methyl-DOPE dispersions containing 2 mol % 1,2-diolein (○), 2 mol % 1,3-diolein (●), and 2 mol % eicosane (□). The initial leakage rate was determined graphically from the time-dependent leakage profiles.

The NBD-PE/Rh-PE RET assays are a measure of membrane fusion which are not affected by vesicle leakage. The rate of lipid intermixing for *N*-methyl-DOPE vesicles containing either 1,2- or 1,3-diolein at 30 °C is shown in Figure 7a. Vesicle fusion can also be measured by the ANTS/DPX assay using the more stringent criterion of the mixing of aqueous contents. The ANTS/DPX assays were less reliable at high temperatures due to the large extent of leakage in the *N*-methyl-DOPE lipid systems. At 30 °C, little leakage was observed, and the time-dependent fusion profiles at 30 °C of *N*-methyl-DOPE with 2% 1,2-diolein or 1,3-diolein indicate faster fusion in the presence of the former lipid additive (Figure 7b).

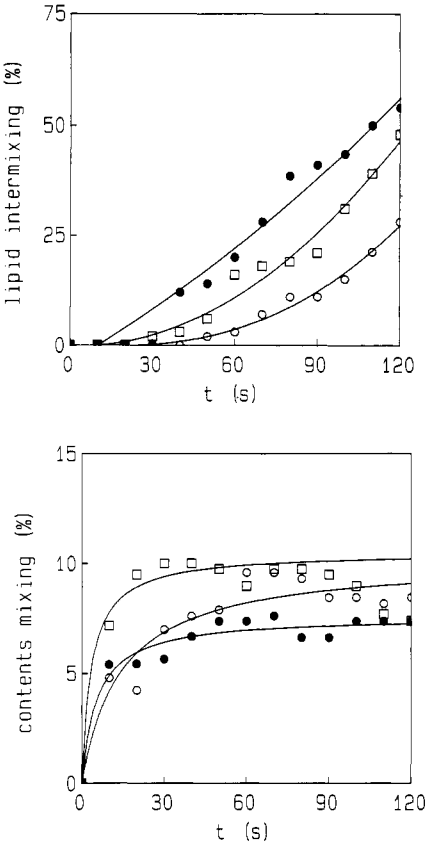

FIGURE 7: (a, top) Time-dependent lipid intermixing at 30 °C for *N*-methyl-DOPE dispersions containing 2 mol % 1,2-diolein (□), 1,3-diolein (○), or eicosane (●). (b, bottom) Time-dependent aqueous contents mixing at 30 °C for *N*-methyl-DOPE dispersions containing 1,2-diolein (□), 1,3-diolein (○), or eicosane (●).

DISCUSSION

It has been suggested that the formation of cubic arrays in the *N*-methyl-DOPE liposomes occurs via high-curvature intermediates such as interlamellar attachments (ILA) and/or inverted micellar intermediates (IMI) (Ellens et al., 1989; Siegel et al., 1989a). The formation of ordered cubic arrays is very slow (Gruner et al., 1988), although the presence of isotropic resonance by ^{31}P NMR spectroscopy (Ellens et al., 1989; Siegel et al., 1989a; Gagne et al., 1985) and disordered cubic phases by X-ray diffraction (Gruner et al., 1988) can be readily observed. Therefore, it is suggested that the observed ^{31}P NMR isotropic phases do not necessary represent highly ordered cubic phases (*Im3m* or *Pn3m*) but may be disordered bilayer intermediates or regions of cubic phases without long-range order.

X-ray studies have shown that the amount of this disordered phase increases with time (Gruner et al., 1988). The time evolution of the isotropic phase in ^{31}P NMR was not studied (Siegel et al., 1989a,b). During the time-dependent NMR experiments described herein, no changes in hydration or lipid degradation were expected because this would also influence the L_α–H_{II} transition temperature drastically, which was not observed. Even at 66 °C the L_α phase was observed and converted into an isotropic phase but not into a hexagonal array.

The temperature at which the isotropic ^{31}P NMR phase first appears, T_I, depends on the history/age of the sample as well as the purity of the synthetic lipid. An isotropic phase could be observed in pure *N*-methyl-DOPE as low as 40–45 °C. Hydrophobic impurities, lipid degradation products, and incomplete hydration lower T_I significantly and also decreases T_H. Our samples have a 40-fold molar excess of water and are therefore likely to be fully hydrated. Increasing the amount of excess water has no effect on the phase transition temperatures. Suspensions of *N*-methyl-DOPE which have been cycled several times through T_H and maintained several hours at high temperatures often show lipid degradation products. Data reported in this paper were obtained with fresh lipid suspensions which were hydrated by freeze–thaw cycling and an equilibration period after each temperature increment. These samples show no lipid degradation (except for acyl chain migration of 1,2-diolein at higher temperatures) and are highly reproducible. In the literature, there is a large discrepancy with regard to the temperature at which the isotropic phase was first observed and the amount of this phase at any given temperature (Ellens et al., 1989; Siegel et al., 1989a; Gagne et al., 1985). The ^{31}P NMR spectra depend on the experimental conditions and especially on the temperature equilibrations around the L_α–H_{II} phase transition, and therefore some of the differences in the reported results likely arise from systematic differences in the protocols used. In *N*-methyl-DOPE, a temperature overshoot into the H_{II} phase and subsequent equilibration below T_H will result in a large increase in the isotropic phase with respect to the bilayer powder pattern due to the metastability of the isotropic phase.

Although the isotropic phase is observed at lower temperatures in the presence of bilayer destabilizers, the extent of the isotropic phase formation is usually much less. Another observation is that some reversibility between isotropic and bilayer phases does occur in the presence of the additives. The most striking difference between the pure lipid system and in the presence of additives is that the addition of bilayer destabilizers results in no increase in the isotropic phase above T_H. For *N*-methyl-DOPE + 2% 1,2-diolein at 40 and 45 °C, three-component ^{31}P NMR spectra, consisting of bilayer L_α,

isotropic, and hexagonal H_{II} phases, were observed. At 45 °C, the L_α phase converted rapidly into an isotropic phase, but the spectral intensity arising from the hexagonal phase only decreased slowly. This indicates that these additives promote the kinetic stabilization of the hexagonal phase. The broad L_α–H_{II} phase transition overlaps with the bilayer to isotropic phase transition and results in the formation of hexagonal phases at the cost of isotropic cubic arrays. Thus, with pure *N*-methyl-DOPE, the isotropic phase can form at temperatures lower than that required for hexagonal phase formation. However, in the presence of additives, the broadened transition to the hexagonal phase overlaps T_I and results in less metastable isotropic phase being formed because of much more rapid equilibration. The hexagonal phase is formed instantaneously compared with the slow formation rate of the isotropic phase and is therefore kinetically preferred. In molecularly heterogeneous lipid dispersions, such as egg PE, no isotropic phases are observed (Cullis & de Kruijff, 1978). Presumably, the broader bilayer–H_{II} transition overlaps T_I, and the lipid is immediately converted into the hexagonal phase.

The initial rate of formation of the isotropic ^{31}P NMR phase may be correlated with a residual temperature, T_H–T, where T is the temperature at which the rate is being measured. The initial formation rates of the isotropic phase at a particular residual temperature are overlapping for all systems studied.

The phase behavior described above is for multilamellar vesicles. Leakage and fusion rates cannot readily be accurately measured with such lipid preparations but rather require unilamellar vesicles. In order for the LUV's used for the leakage and fusion studies to be converted into isotropic or H_{II} phases requires vesicle–vesicle contact. Therefore, the phase bahavior of the LUV's may not correspond exactly to the phase behavior we measured by DSC and ^{31}P NMR using multilamellar preparations. Thus, although a small amount of structures which give rise to isotropic ^{31}P NMR signals may be accomodated within the bilayer, extensive formation of nonbilayer phases would result in the total release of vesicle contents. As a result, vesicle fusion would be observed by lipid intermixing but not by the mixing of internal contents.

It had been suggested that the rate of membrane fusion is correlated with the amount of isotropic phase present (Ellens et al., 1989). We find that the size of the isotropic peak increases with time but the rate of leakage or fusion does not increase with time. In addition, dioleins or eicosane, which increase the rate of fusion, decrease the extent of formation of isotropic ^{31}P NMR signals because they favor H_{II} phase formation. In addition, there is significant membrane fusion at 30 °C in the presence of destabilizers, even though there is no detectable isotropic phase at this temperature by ^{31}P NMR. Furthermore, there is no marked increase in leakage rates between 30 and 40 °C despite the fact that isotropic phases begin to appear in this temperature range. However, there is a correlation between the temperature at which isotropic or H_{II} phases are formed and the rate of leakage and fusion. Addition of eicosane or diolein causes both a lowering of T_I and T_H as well as an increase in the rates of membrane leakage and fusion. This suggests that bilayer destabilization or the formation of nonspecific defects is primarily responsible for the observed increase in the rates of leakage and fusion, rather than the formation of specific intermediates. This does not rule out the possibility that specific structures such as ILA's are intermediates in membrane fusion, as has been observed by cryotransmission electron microscopy (Siegel et al., 1989c). It would suggest, however, that the rate-determining step in membrane fusion occurs prior to the formation of such intermediates. On a longer time scale, a good correlation was found between isotropic ^{31}P NMR signals from LUV's and the initial rate of fusion (Kelsey et al., 1991). Membrane fusion can be described kinetically as a reversible association followed by an irreversible fusion step (Bentz et al., 1983). No kinetic analysis of the effects of dioleins has been performed, and it is possible that they promote vesicle aggregation rather than fusion.

As discussed above, we have chosen membrane additives which would favor the H_{II} phase over the L_α phase by different mechanisms. Thus, the dioleins would affect intrinsic bilayer curvature, while eicosane would primarily stabilize the H_{II} phase by relieving hydrocarbon packing constraints. Despite this difference, both the dioleins and eicosane have similar effects on membrane phase behavior and on leakage and fusion rates.

Although the effects of these additives are similar, they are not identical. For example, 1,2-diolein consistently has somewhat greater effects in destabilizing the bilayer phase than the structurally similar 1,3-diolein. This is the case for the appearance of isotropic ^{31}P NMR signals and for leakage and fusion rates. The 1,2-diolein is a potent activator of PKC. Uncharged or zwitterionic compounds that affect PKC activity, including eicosane (Epand et al., 1988), are activators of the enzyme if they lower T_H (Epand & Lester, 1990). But not all such bilayer destabilizers are potent modulators of PKC activity (Epand et al., 1989). In the case of diolein, both the 1,2- and 1,3-isomers are potent hexagonal phase promoters, yet only the 1,2-isomer is effective in activating the enzyme (Boni & Rando, 1985; Molleyres & Rando, 1988). It is likely that most of the discrimination between the two isomers is a result of their different interaction with the enzyme. Not all isoforms of PKC behave identically with the diolein isomers, further complicating the correlation between the properties of the activators and their effects on the activity of PKC. Type II PKC is activated by 1,3-diolein as well as 1,2-diolein (Allen & Katz, 1991). However, for the majority of PKC isoforms, as exhibited by unfractionated PKC, only the 1,2-isomers can activate. It is possible that, in addition to the discrimination among effectors of PKC activity which occurs at the level of protein binding, effects of dioleins on membrane physical properties could also contribute to their modulation of PKC activity. Thus, despite the chemical similarities between 1,2- and 1,3-diolein, the two compounds have different structural and physical properties. The two isomers of diolein have different crystal structures. The 1,3-diolein has the acyl chains on either side of the glycerol backbone, while the 1,2-isomer has parallel acyl chains on one side of the head group. However, the differences in the membrane are less pronounced (Hamilton et al., 1991a,b). In PC bilayers, the glycerol backbone of the 1,2-diolein has an orientation similar to that in the phospholipid and is parallel with both acyl chains, resulting in a larger hydrogen-bonding capacity for the *sn*-2 carbonyl. In the 1,3-isomer, the glycerol backbone is situated normal to the acyl chains, and both carbonyls have an equivalent hydrogen-bonding capacity. The difference in orientation of the glycerol backbone in the 1,2- and 1,3-isomer of diolein may result in the lack of PKC activation for 1,3-diolein and PKC activation in the presence of 1,2-diolein as a result of differences in the structural properties of the dioleins. These differences may also result in their different effects on membrane physical properties. The 1,2-diolein is somewhat more effective than the 1,3-isomer in lowering T_H. The 1,2-diolein also has much greater effects on the rate of

membrane fusion at temperatures where the membrane remains in the bilayer phase. Thus, despite of their similar structure, the two diolein isomers affect membrane phase behavior and vesicle fusion and leakage rates to different extents. These properties are likely altered by changes in the physical properties of the membranes. Thus, 1,2-diolein is more effective in destabilizing bilayers than is the 1,3-isomer. This may contribute to its greater ability to activate PKC.

Registry No. *N*-Methyl-DOPE, 96687-23-9; 1,2-diolein, 2442-61-7; 1,3-diolein, 2465-32-9; eicosane, 112-95-8.

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Structure of Epidermal Growth Factor Bound to Perdeuterated Dodecylphosphocholine Micelles Determined by Two-Dimensional NMR and Simulated Annealing Calculations†

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ABSTRACT: The interaction of mouse epidermal growth factor (mEGF) with micelles of a phospholipid analogue, perdeuterated dodecylphosphocholine (DPC), was investigated by two-dimensional ^{1}H NMR. Sequence-specific resonance assignments of the micelle-bound mEGF have been made, and the chemical shifts were compared with those in the absence of DPC. DPC induced large chemical shift changes of the resonances from the residues in the C-terminal tail (residues 46-53) but little perturbation on the residues in the main core (residues 1-45). Starting from the three-dimensional structure in the absence of DPC, micelle-bound structures were calculated using the program XPLOR with interproton distance data obtained from NOESY spectra recorded in the presence of DPC. The C-terminal tail of mEGF was found to change conformation to form an amphiphilic structure when bound to the micelles. It is possible that induced fit in the C-terminal tail of mEGF occurs upon binding to a putative hydrophobic pocket of the EGF receptor.

Micellar systems have long been used as models for membranes. They are superior to organic solvent systems, such as methanol and trifluoroethanol, since they can mimic the lipid-water interface as well as the hydrophobic environment. In the field of ^{1}H NMR, the conformations of peptides bound to micelles have been investigated as a model of membrane-bound forms (Brown et al., 1982; Braun et al., 1983; Olejniczak et al., 1988; Inagaki et al., 1989; Endo et al., 1989).

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