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Lipid-Polyethylene Glycol Interactions: II. Formation of Defects in Bilayers

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Summary. Polyethylene glycol, a known cell fusogen, is found to induce the formation of structural defects in egg phosphatidylcholine multilamellar vesicles, as shown by freeze-fracture microscopy. ^{31}P NMR spectra of these vesicles reveal the existence of a nonbilayer (isotropic) phase. The observed disruption in the bilayers is believed to be associated with an intermediate stage of membrane fusion.

Key words: Polyethylene glycol, bilayer defects, multilamellar vesicles, ^{31}P NMR, freeze fracture, X-ray diffraction

Abbreviations

PEG – Polyethylene glycol
IMP – Intramembranous particle
PC – Phosphatidylcholine
PS – Phosphatidylserine
SUV – Small unilamellar vesicles
MLV – Multilamellar vesicles
DPPC – Dipalmitoyl phosphatidylcholine
DSC – Differential scanning calorimetry
DMPC – Dimyristoylphosphatidylcholine
T_c – Phase transition temperature

The arrangement of lipids in a bilayer configuration in biological and model membranes is well accepted (Wilkins, Blaurock, & Engelman, 1971). However, the presence of a transient nonbilayer configuration is required at some intermediate step during membrane fusion (Lucy, 1970; Papahadjopoulos, Poste, Schaeffler, & Vail, 1974; Lawaczeck, Kainosho, & Chan, 1976; Cullis & Hope, 1978; Prestegard & Kantor, 1978; Verkleij et al., 1979). It has been suggested (Verkleij et al., 1979) that a form of bilayer instability, namely lipidic particles, appear at the fusion sites of egg phosphatidylcholine-cardiolipin vesicles when calcium is added to induce fusion. The appearance of lipidic particles has been associated with an isotropic phase in ^{31}P NMR spectra. This isotropic phase is usually superimposed on the broader, anisotropic phase that is found for lipids in a bilayer configuration (Cullis & de Kruijff, 1979). Examples of this correlation between lipidic particles and an isotropic phase has been seen in both synthetic membranes (Verkleij et al., 1979; Gerritsen et al., 1980) and biological membranes (de Kruijff et al., 1978; Burnell, van Alphen, Verkleij & de Kruijff, 1980; Stier, Finch & Bosterling, 1978). It appears that bilayer instabilities are not uncommon in membranes, especially in connection with membrane fusion.

In part I of this study (Boni, Stewart, Alderfer & Hui, 1981) we have shown that PEG can induce fusion between small unilamellar vesicles (SUV). In this section we have investigated the effects of PEG on the structure of egg PC multilamellar vesicles by freeze-fracture electron microscopy, ^{31}P NMR, and X-ray diffraction in an effort to observe any associated nonbilayer configurations in connection with the fusogenic effect of PEG.

Materials and Methods

Vesicle Preparation

Dimyristoyl phosphatidylcholine was obtained from Sigma Chemical Co., St. Louis, Mo. Other lipids used were described in part I (Boni et al., 1981). MLV of these lipids were prepared as outlined in Boni et al. (1981). Pelleted MLV were resuspended in 1.5 ml of the appropriate concentration of PEG. Mixing was obtained by vigorous shaking and vortexing. Egg PC samples in 50% PEG were either placed in 10-mm NMR tubes for spectral analysis or used immediately for freeze fracture. The samples were further concentrated by centrifugation at 20,000 × g for 1 hr. The concentrated lipid floated on the media containing PEG and was collected for X-ray diffraction analysis. The procedures for freeze-fracture electron microscopy and X-ray diffraction were given in Boni et al. (1981).

^{31}P NMR

A Bruker WP-200 Fourier transform spectrometer was used for this study. ^{31}P NMR spectra were acquired at 81 MHz, using an

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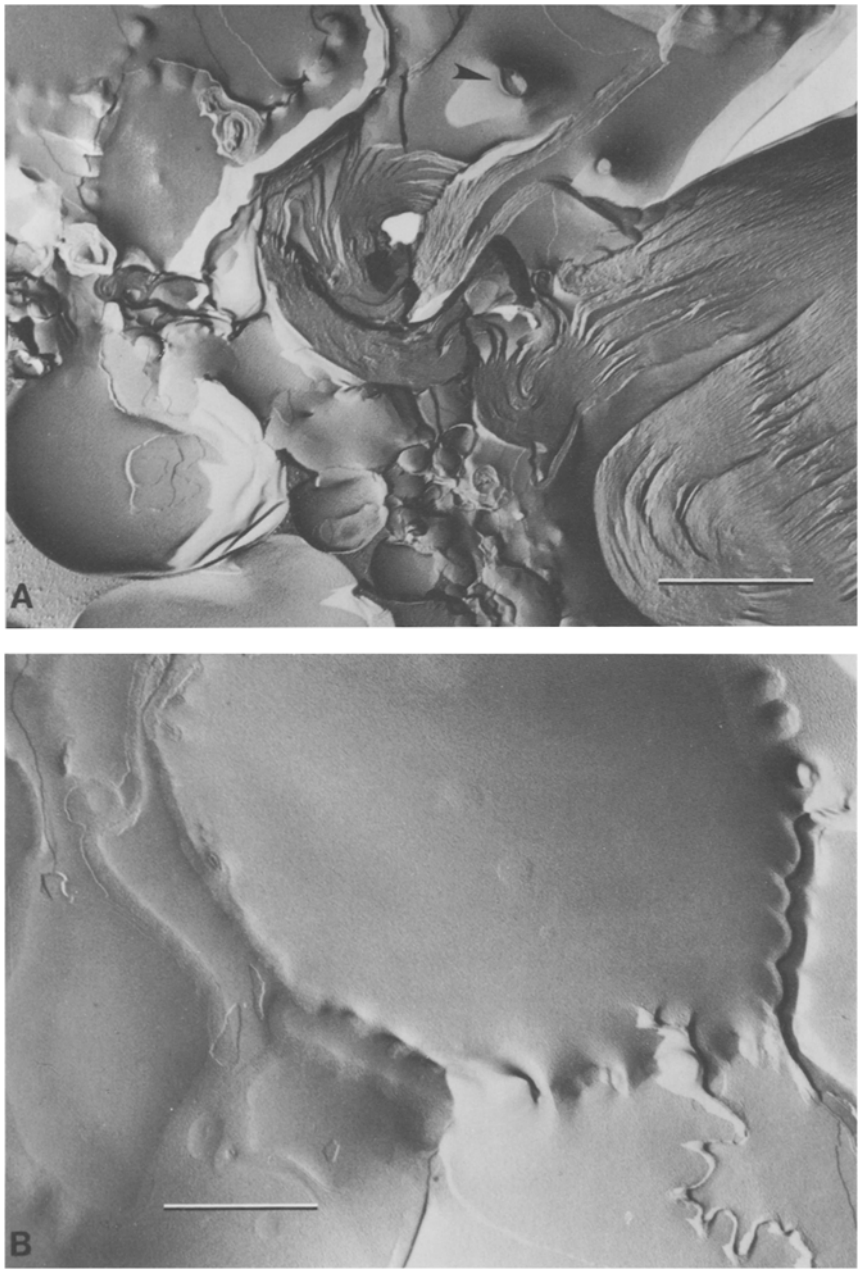

Fig. 1. Freeze-fracture electron micrographs of egg phosphatidylcholine MLV in 50% PEG, illustrating various bilayer defects. All show a textured surface. (*A*): Tightly packed bilayers with various peaks and pits including broken tips (arrow). (*B*): A continuous link of tightly apposed areas and scalloped surfaces.

inverted gated decoupling sequence (i.e., proton decoupling with NOE suppression) with a 7-sec delay between pulses. The lipid concentration was approximately 20 mM in all experiments. Up to 600 transients were taken for each spectrum. Exponential filtering was employed for signal enhancement (a line broadening of 200 Hz).

The saturation transfer experiment was performed using the DANTE pulse train of Morris and Freeman (1978). The sequence employed was, following de Kruijff, Morris, and Cullis (1980), [D1-(P1-D2)Np-P1-D3-Pw-De-Acquisition]×Ns. The numerical values were D1=3 sec; P1=0.4 μsec; D2=D3=1 msec; De=10 msec; Pw=15 μsec; Np=10,000; Ns=1,500, with an acquisition time of 0.1 sec.

Results

Freeze-fracture electron micrographs of egg PC MLV in 50% PEG revealed numerous features as illustrated in Fig. 1. The fractured surfaces are seen to have a worm-like texture rather than being smooth as in egg PC vesicles in excess water (Ranck et al., 1974; Kleman, Williams, Costello, & Gulik-Krzywicki, 1977). This texture is identical to that found on the MLV formed when SUV are mixed in 50% PEG (Boni et al., 1981). Many conical protrusions, peaks, and pits (bilayer defects of high curvature), of diame-

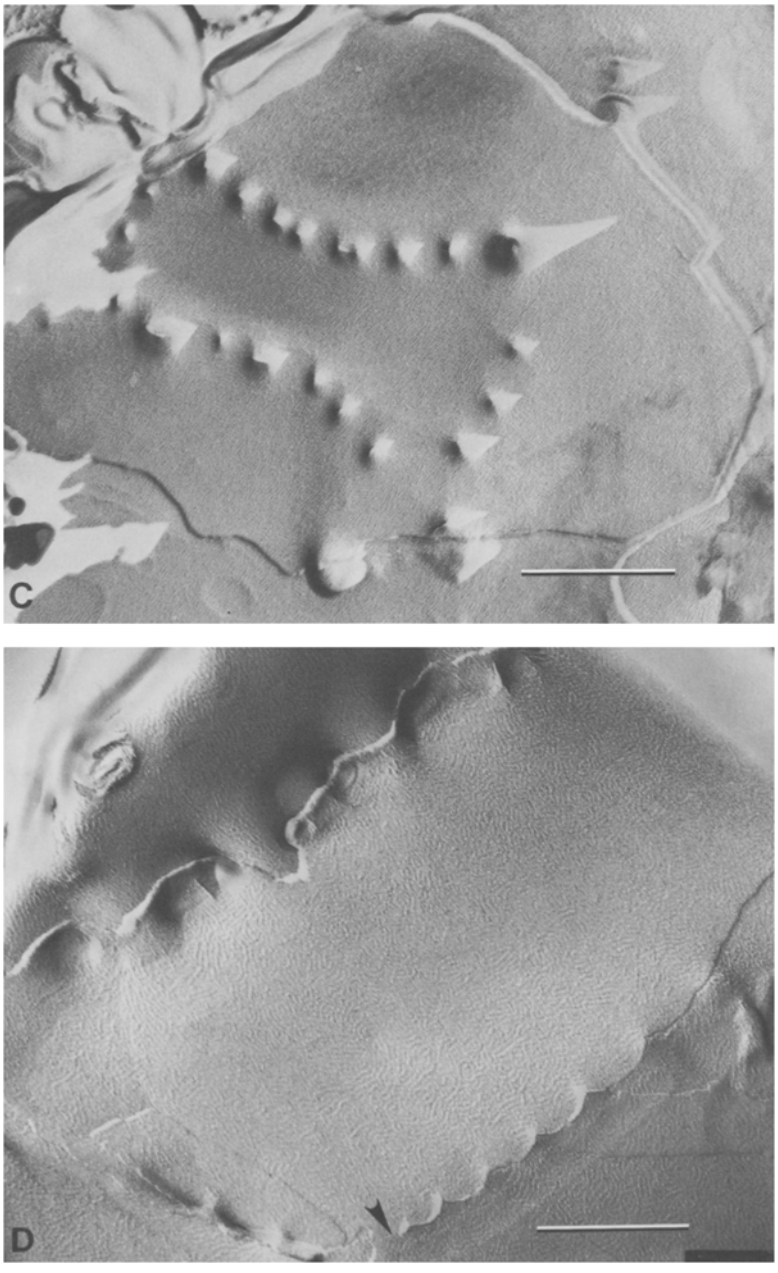

Fig. 1. (*C*): Rows of peaks and pits. (*D*): A scalloped fracture line ending in a screw dislocation (arrow).

ters from 100 to 1,000 nm can be seen on the fracture faces of these MLV. Some of these defects are seen to be aligned in evenly spaced rows (Fig. 1 *c*). What appears to be a screw dislocation or a link between adjacent bilayers is shown in Fig. 1 *d* (arrowed). In broken tips of conical protrusions, concentric layers can be seen to converge to a point (Fig. 1 *a* and *e*). Rows of tightly attached areas between bilayers are also discernable. Some of these areas form a scalloped-type arrangement with protrusions along the edges (Fig. 1 *b* and *f*).

X-ray diffraction patterns of egg PC in 50% PEG consist of at least three orders of low angle lamellar repeat spacings, which decrease as the temperature is increased (Fig. 2). The sharp, low angle spacings represent lamellar repeats. A very diffuse diffraction band corresponding to repeat spacings in the range of 90–200 Å is also observed, most likely due to the worm-like texture on the surfaces. No sharp wide angle reflections were noted, even at −5 °C.

Further information on the nature of the phospholipids in the bilayers were obtained through ^{31}P NMR

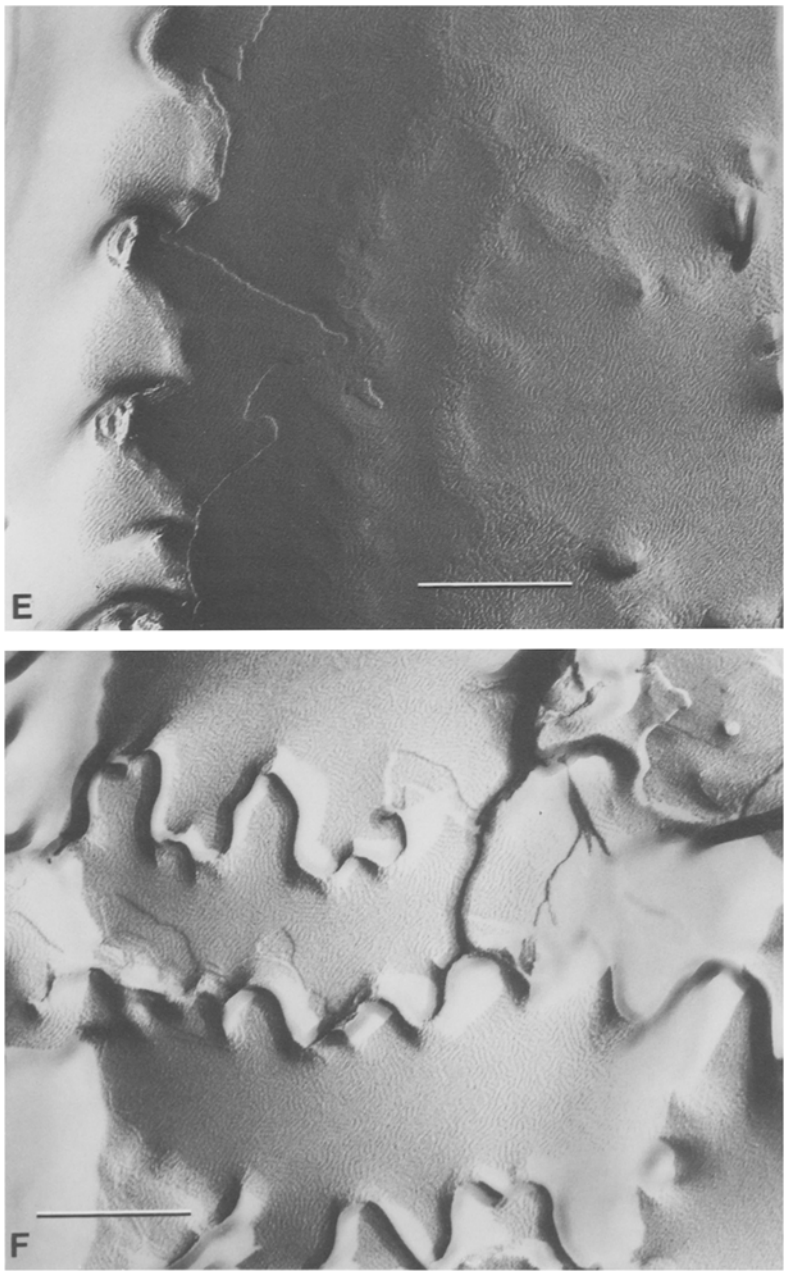

Fig. 1. (*E*): Broken tips of elongated peaks which contain several concentric bilayers, possibly a fracture through merging layers between neighboring confocal domains. (*F*): Scalloped deformations. Bar = 0.6 μm in *a* and *b* and 0.3 μm in *c–f*

studies. In mixtures of egg PC with increasing concentrations of PEG (Fig. 3), a resonance appears with a chemical shift and shape characteristic of an isotropic motional averaging. The presence of this isotropic resonance indicates that there is rapid molecular motional averaging in the sample, with the proportion of molecules participating in the motional averaging increasing with increasing PEG concentrations. Similar sharp resonances were obtained for MLV in 50% PEG for DMPC, bovine phosphatidylserine, soybean phosphatidylethanolamine, and human erythrocyte ghost membranes (*unpublished results*).

In an attempt to locate the nature of the phospholipid components that give rise to the isotropic phase, an excess of the shift reagent praeseodynium chloride at a molar ratio of Pr^{3+}/phosphate = 0.14 (Hutton, Yeagle & Martin, 1977) was added to the PC MLV-50% PEG mixture. The presence of this shift reagent is expected to cause a downfield shift of ^{31}P resonances of those phospholipids whose head groups are exposed to the aqueous medium. No observable spectral change was noted.

Application of the DANTE saturation transfer technique was employed to determine the rate at

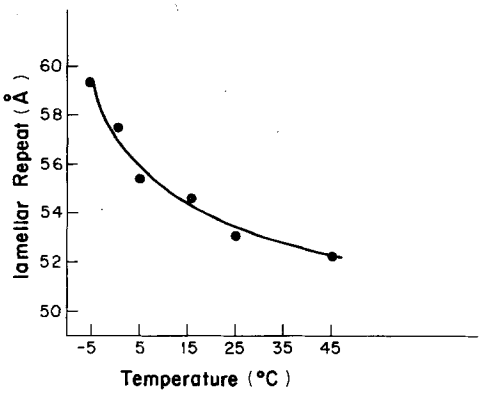

Fig. 2. The change in lamellar repeat spacings *vs.* temperature for egg phosphatidylcholine MLV in 50% PEG

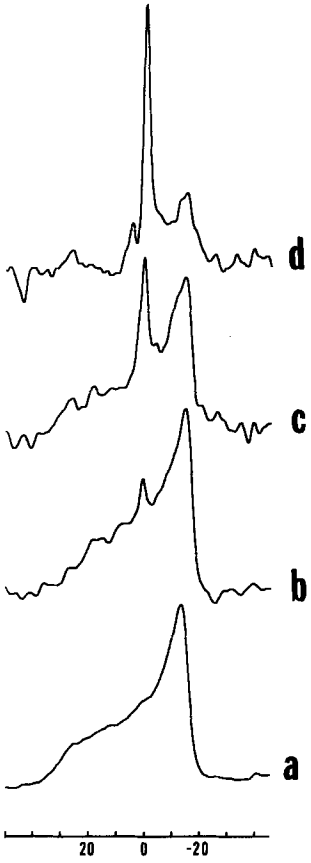

Fig. 3. 81 MHz ^{31}P NMR spectra of egg phosphatidylcholine MLV in various PEG percentages (wt/wt), revealing an increase in the isotropic resonance with increasing percent PEG. Spectra were taken at 30 °C except for egg PC in 67% PEG, which was taken at 50 °C. (*a*) Pure MLV; (*b*) MLV in 30% PEG; (*c*) MLV in 50% PEG; and (*d*) MLV in 67% PEG

which phospholipids in regions causing the isotropic phase interchange with those phospholipids in the bilayer phase. If the exchange rate is fast relative to the saturation time and the spin-lattice relaxation time, the saturation transfer will cause both resonances to disappear. The results are shown in Fig. 4.

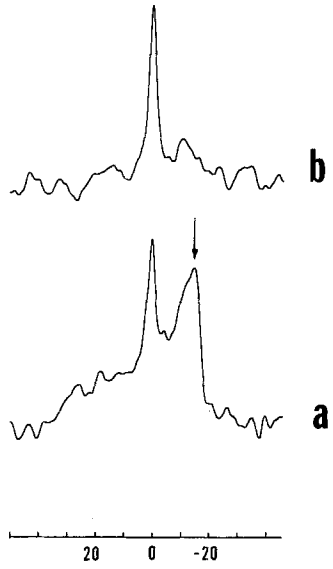

Fig. 4. Saturation transfer ^{31}P NMR experiment on egg PC in 50% PEG, employing the DANTE pulse sequence (*see* Materials and Methods). The arrow indicates the frequency at which the pulse train was applied. (*a*): Spectrum of egg PC MLV in 50% PEG. (*b*): Illustrates the effect of the saturation. Spectra were recorded at 30 °C

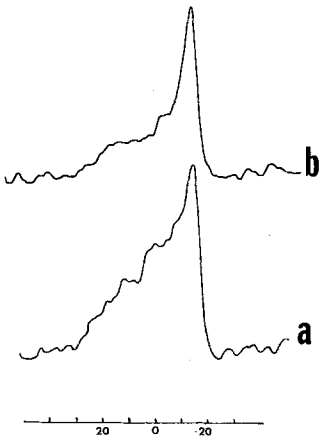

Fig. 5. 81 MHz ^{31}P NMR spectra of egg phosphatidylcholine MLV in (*a*) 25% dextran (mol wt 500,000) at 50 °C and (*b*) in 85% glycerol at 30 °C

The inability to reduce the isotropic resonance by saturating the bilayer resonance (arrow), along with the fact that the relaxation times for MLV in 50% PEG are between 1 and 2 sec (*unpublished results*), indicates that the lifetime of the phospholipids in these two phases is greater than 1 sec.

To determine whether this isotropic phase was solely due to dehydration of the bilayer, 25% dextran was added to egg PC MLV at 50 °C. In contrast to PEG, the dextran only slightly broadened the resonance, but yielded no evidence of an isotropic phase (Fig. 5*a*). No significant change was noted for egg PC MLV in 85% glycerin (Fig. 5*b*), another dehydrat-

ing agent, as compared to the spectrum of pure egg PC MLV. Moreover, although MLV suspensions of PC at low (~15%) water content contained many similar defect structures (Kleman et al., 1977), no isotropic ^{31}P NMR signal was observed in comparable specimens (de Kruijff et al., 1980). It should be noted that slightly hydrated MLV suspensions give rise to an anisotropic ^{31}P NMR resonance that is broadened by approximately 15 ppm as compared to fully hydrated MLV (de Kruijff et al., 1980). We did not observe any broadening in the resonances of egg PC MLV in PEG as compared to the fully hydrated spectrum (Fig. 3). In addition, while the anisotropic resonance of PC bilayers in 50% PEG was completely obliterated by the saturation transfer experiment, applying this technique to slightly hydrated MLV only resulted in "hole burning" (de Kruijff et al., 1980). Assuming similar relaxation times, this implies a greater mobility for the phospholipid molecules in MLV immersed in PEG than those in dehydrated MLV. In any case, bilayers in PEG are not simply dehydrated.

Discussion

Approximately all the available water is bound to the polymer at a PEG concentration of 50% wt/wt (Baran, Solomentseva, Mank & Kurilenko, 1972; Blow et al., 1978), resulting in a highly dehydrated MLV suspension. This is evidenced by freeze-fracture electron microscopy and X-ray diffraction. Freeze-fracture micrographs reveal a worm-like pattern, analogous to that found for egg PC dispersions that are 10–20% hydrated (Ranck et al., 1974; Kleman et al., 1977). The X-ray lamellar repeat spacings (Fig. 2) also indicate smaller than the normal repeat spacings (63 Å) expected for fully hydrated egg PC MLV (Shipley, 1973), corresponding to the spacings of dehydrated phases (Ranck et al., 1974). The repeat spacings in PEG-containing samples also correspond to the lamellar repeat spacings found when MLV are suspended in dextran, another dehydrating agent (LeNeveu, Rand, Parsegian & Gingell, 1977). The dehydration effect of PEG is further evidenced by comparing the morphology seen in our freeze-fracture micrographs (Fig. 1) and that of egg PC in 16% water (Kleman et al., 1977), both showing evenly spaced peaks and pits in rows. These were attributed to a relaxation strain between large confocal domains (*see* Fig. 1 of Kleman et al., 1977). The core of a confocal domain (de Gennes, 1974) along the hyperbolic axis connects the hydrophobic regions of distant layers.

Differential scanning calorimetry (DSC) exotherms of dipalmitoyl phosphatidylcholine (DPPC) MLV in 50% PEG show both an increase in the transition temperature (T_c) and a broadening of the peaks (Tilcock & Fisher, 1979). We have obtained similar results for egg PC MLV in 50% PEG and DMPC MLV in 50% PEG (results not shown). The increase in T_c is consistent with the phospholipids being dehydrated (Ladbrooke & Chapman, 1969). The broadening is indicative of decreasing cooperativity in the acyl chains (Marsh, Watts & Knowles, 1977), as would be expected from packing distortions in the bilayer.

^{31}P NMR spectra clearly indicates the existence of nonbilayer configurations induced by PEG in egg PC MLV. This is manifested in the existence of the isotropic resonances. That DMPC MLV, with homogenous acyl chains, gives rise to similar spectra indicates that sequestering into domains of phospholipids of different acyl chain content (with respect to length and degree of unsaturation) is not the cause of the isotropic resonances. This isotropic phase does not seem to be a result of aqueous suspensions of small lipid vesicles or micelles (Satir, Schooley & Satir, 1973; Knutton, 1979), as revealed by the absence of any broadening or shifting of the isotropic peak upon addition of excess shift reagent. It is of interest to note that the isotropic ^{31}P nuclear magnetic resonance was also observed in a number of lipid mixtures exhibiting cusp-shaped "lipidic particles" on freeze-fracture faces (de Kruijff et al., 1979; Verkleij et al., 1979; Gerritsen et al., 1980; Hui, Stewart, Yeagle & Albert, 1981). The cause of the isotropic phase in these systems has been attributed to motional averaging arising from free rotation via lateral diffusion about the highly curved surfaces such as invaginations or inverted micelles (Verkleij et al., 1979; Stier et al., 1978; Cullis & de Kruijff, 1979). These nonbilayer structures have been associated with membrane fusion (Verkleij et al., 1979). It is also of importance to note that PEG is unique with respect to other dehydrating agents. Dextran is nonfusogenic (Ahkong, Fisher, Tampion & Lucy, 1975) and as shown in Fig. 5 did not give rise to a ^{31}P NMR isotropic resonance. Similar NMR results were obtained with glycerol. This supports the argument that PEG has additional microscopic properties, such as its ability to solvate the polar head group (Tilcock & Fisher, 1979), and alter the electrostatic interactions between bilayers.

We have shown that polyethylene glycol, which is commonly used as a cell fusogen, produces many bilayer defects. There is a definite association between these defects with the appearance of an isotropic ^{31}P NMR resonance and that the molecules in these defects are sequestered from the bilayer and are not accessible to any aqueous environment. These nonbilayer configurations, particularly the peaks and pits shown in the freeze-fracture micrographs, are similar

to bilayer defects found in natural and synthetic membranes under conditions favorable for fusion. In view of the fusogenic effect of PEG, it is plausible to consider these observed bilayer defects created in the presense of PEG as representing an arrested intermediate stage of bilayer fusion.

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