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Transbilayer Distribution of Phosphatidylethanolamine in Large and Small Unilamellar Vesicles†

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ABSTRACT: There is much evidence which strongly suggests that most constituents of biological membranes display a transbilayer compositional asymmetry. The tendency of binary mixtures of phospholipids to form compositionally asymmetric bilayers spontaneously has been studied extensively. In small unilamellar vesicles, most mixtures of phospholipids with different head groups have been reported to be nonrandomly arranged across the bilayer. In this study, the influence of the radius of curvature on the transbilayer phospholipid distribution has been investigated. The distribution of egg phosphatidylethanolamine in large unilamellar vesicles comprised of egg phosphatidylethanolamine and egg phosphatidylcholine was determined by 2,4,6-trinitrobenzenesulfonic acid labeling. These large vesicles were obtained by modifying the ethanol injection procedure originally described by Batzri & Korn (1973) [Batzri, S., & Korn, E. D. (1973) *Biochim. Biophys. Acta 298*, 1015] by using a slow injection rate. After injection, the ethanol was removed by molecular sieve chromatography and the vesicle dispersion centrifuged. This results in a population of large, homogeneous, and unilamellar vesicles as determined by molecular sieve chromatography, ^{31}P NMR, and electron microscopy. The phosphatidylethanolamine component in unilamellar vesicles of this type is equally distributed between the two monolayers. In contrast, phosphatidylethanolamine in small unilamellar vesicles is known to be preferentially localized in the outer monolayer at low phosphatidylethanolamine concentrations and in the inner monolayer at high phosphatidylethanolamine concentrations. These results suggest that while phospholipids may form asymmetric bilayers spontaneously in highly curved regions of biological membranes, other factors must be responsible for the generalized phospholipid asymmetry seen in these systems.

It is generally believed that biological membranes are vectorial structures. Presumably, the different chemical environments and functional requirements found on opposing sides of the membrane dictate the arrangement of the membrane components. For example, all data indicate an absolute asymmetry in the orientation of membrane-bound proteins (Rothman & Lenard, 1977). Proteins that partially penetrate the membrane or are superficially attached to it are specifically located at one side while membrane-spanning proteins have well-defined orientations within the bilayer. Phospholipids are also distributed asymmetrically across the bilayer, although most phospholipids are present in both monolayers. The most convincing evidence for this has been obtained for erythrocytes. Data acquired by several techniques indicate that phosphatidylcholine and sphingomyelin occupy the outer monolayer whereas phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine are localized in the inner monolayer [reviewed by Op den Kamp (1979)].

Small unilamellar vesicles made by sonication (Huang, 1969) or rapid ethanol injection (Batzri & Korn, 1973) have been widely used as models for biological membranes. The highly curved surfaces characteristic of these vesicles are present in some specialized membrane structures such as the mitochondrial cristae or the microvilli of the intestinal epithelial cells. However, most biological membranes, like the plasma membrane or the delimiting membrane of cellular organelles, are essentially planar, although restricted regions may exhibit small radii of curvature (Thompson et al., 1974).

The utility of small unilamellar vesicles as model membranes must be assessed by their ability to approximate the biological structures they are expected to represent. Since the bilayers comprising these vesicles are highly curved whereas most biological membranes are not, it is important to explore the relationship between the radius of curvature and various bilayer properties. In fact, it has been shown that some physical properties of phospholipids, such as molecular motions (Sheetz & Chan, 1972) and the thermotropic behavior of the phospholipid components (Suurkuusk et al., 1976; Lentz et al., 1976a,b), are very sensitive to changes in the radius of cur-

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vature. The permeability properties of liposomes and their response to transbilayer osmotic gradients have also been found to depend on the curvature (Barenholz et al., 1979; Lichtenberg et al., 1981).

Recently, several investigators have reported that binary mixtures of phospholipids will form compositionally asymmetric bilayers spontaneously (Litman, 1973, 1974, 1975; Berden et al., 1975; Michaelson et al., 1973; Lentz et al., 1980; Massari et al., 1978). It has been postulated by Israelachvili et al. (1977) that the difference in the molecular packing densities in the inner and outer leaflets of highly curved bilayers (Huang & Mason, 1978; Chrzeszczyk et al., 1977) may cause phospholipids to be asymmetrically distributed across the bilayer. For example, the asymmetric distribution of phosphatidylethanolamine in small unilamellar vesicles composed of this phospholipid and phosphatidylcholine seems to be affected by the acyl side chain composition and the packing requirements of the head groups. Although egg phosphatidylethanolamine in vesicles composed of this lipid and egg phosphatidylcholine was found preferentially in the outer monolayer at low concentrations and in the inner monolayer at high phosphatidylethanolamine concentrations (Litman, 1973, 1974), the acyl side chains were randomly distributed within each phospholipid group (Litman, 1975). However, in contrast to this situation, the inner monolayer is not appreciably enriched with phosphatidylethanolamine in dimyristoylphosphatidylethanolamine–dimyristoylphosphatidylcholine small unilamellar vesicles at any mole percent composition, indicating that the properties of the acyl side chain constituents can in fact influence the transbilayer distribution of the head groups (Lentz & Litman, 1978). Phosphatidylinositol and phosphatidylserine have also been found to be asymmetrically distributed when mixed with egg phosphatidylcholine (Berden et al., 1975). Since small unilamellar vesicles and biological membranes both exhibit phospholipid compositional asymmetry, we have examined the influence of the radius of curvature on the transbilayer phospholipid distribution by comparing the distribution of lipid components in unilamellar vesicles of large radius made by a slow ethanol injection procedure with small unilamellar vesicles made by sonication and rapid ethanol injection. In contrast to the results for vesicles with highly curved bilayers, phosphatidylethanolamine in large unilamellar vesicles formed from this lipid and egg phosphatidylcholine was equally distributed between the two monolayers over a wide composition range. The absence of phospholipid asymmetry in large vesicles suggests that other membrane constituents must play an important role in the formation and retention of phospholipid asymmetry in biological membranes. Thus, studies concerning membrane asymmetry will yield the most information of relevance to biological membranes if they are pursued in vesicles having very little surface curvature.

Materials and Methods

Phospholipid Preparation. Egg phosphatidylcholine (PC)[1] and egg phosphatidylethanolamine (PE) were purchased from Avanti Biochemicals (Birmingham, AL) and used without further purification. No detectable impurities were observed when the phospholipids (1 μmol) were loaded on thin-layer silicic acid chromatography plates and developed in $CHCl_3$–CH_3OH–H_2O (65:25:4). The phospholipids were dissolved in $CHCl_3$ and stored under argon at −20 °C. The purity of the phospholipids remained unchanged throughout the course of these experiments under these storage conditions.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were prepared by probe sonication and rapid ethanol injection. Mixtures of egg PE and egg PC were evaporated to dryness under argon and lyophilized for 3–4 h in the dark. Phospholipids used to form sonicated SUV were suspended in 100 mM KCl and subjected to ultrasonic irradiation using a Heat System W-350 sonifier. The phospholipid dispersion (2–3 mL), sealed in a glass tube under a nitrogen atmosphere, was sonicated intermittently at 0 °C for 2–3-min periods. The maximum sonication time was 30 min. SUV were also prepared by a rapid ethanol injection procedure (Batzri & Korn, 1973). Lyophilized phospholipids were dissolved in absolute ethanol (35 μmol of phospholipid/mL) and injected rapidly through a 100-μL Hamilton syringe into rapidly stirring 160 mM KCl. The resulting vesicle dispersion contained less than 5% ethanol by volume. The suspension was concentrated to 1–2 mL on an Amicon ultrafiltration device (Lexington, MA) by using an XM-100 membrane. The rapidly stirring suspension was maintained under nitrogen gas whose pressure did not exceed 25 lb/in.^{2}.

A homogeneous population of SUV was prepared from the crude vesicle dispersion by centrifugation for 60 min at 96500*g* (Ti-50 fixed-angle rotor). Only the vesicles found in region III of the supernatant, as described by Barenholz et al. (1977), were used. The pH of the vesicle preparations was 6.8–7.0.

Preparation of Large Unilamellar Vesicles. Large unilamellar vesicles (LUV) were obtained by modifying the ethanol injection procedure described by Batzri & Korn (1973). Colyophilized mixtures of egg PC and egg PE were dissolved in absolute ethanol (10 μmol of phospholipid/mL) and injected slowly (<3 mL/h) into rapidly stirring 100 mM KCl. The resulting vesicle dispersion contained less than 25% ethanol (v/v). The ethanol was completely removed by passing the vesicles over a Sephadex G-50 column (1.6 × 15 cm). The dispersion was concentrated in an Amicon ultrafiltration cell in the manner previously described for SUV. A homogeneous vesicle population was obtained by centrifugation for 38 min at 96500*g* in a Ti-50 fixed-angle rotor. Only the vesicles found in region III of the supernatant, as described by Barenholz et al. (1977), were used. The pH of the vesicle preparations was 6.8–7.0.

It should be noted that the average vesicle size is very sensitive to the total phospholipid concentration in the initial ethanol solution (Kremer et al., 1977) and to the type of phospholipid used. In general, more saturated phospholipids and mixtures of phospholipids will produce larger vesicles than unsaturated phospholipids at the same total phospholipid concentration in ethanol. The optimum centrifugation conditions required to produce homogeneous vesicle populations must be determined for each system studied (Barenholz et al., 1977).

Determination of Phosphatidylethanolamine Distribution. The ratio of phosphatidylethanolamine in the outer vesicle surface to the total phosphatidylethanolamine content was determined by chemical labeling according to a modification of the procedure originally reported by Litman (1973). The outer vesicle surface phosphatidylethanolamine content was determined as follows. An aliquot of an aqueous vesicle solution, containing not more than 0.25 μmol of PE, was diluted to a final volume of 0.6 mL with the vesicle buffer solution. To this was added 0.2 mL of 0.8 M $NaHCO_3$, pH 8.5. The sample was mixed. A 20-mL aliquot of 1.5% 2,4,6-trinitrobenzenesulfonic acid (TNBS) was added, and the sample was

[1] Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

mixed and incubated in the dark for 20 min at room temperature. After incubation, 0.4 mL of 1.2% Triton X-100 in 1.5 N HCl was added. The sample was mixed and stored in the dark. The absorbance at 410 nm was read within 1 h after acidification. The total phosphatidylethanolamine content was determined in a similar manner. An aliquot of an aqueous vesicle solution, containing not more than 0.25 μmol of PE, was diluted to a final volume of 0.6 mL with the vesicle buffer solution. To this was added 0.2 mL of 1.6% Triton X-100 in 0.8 M $NaHCO_3$, pH 8.5. The sample was then mixed. A 20-μL aliquot of 1.5% TNBS was added; the sample was mixed again and incubated in the dark for 20 min at room temperature. After incubation, 0.4 mL of 0.4% Triton X-100 in 1.5 N HCl was added. The sample was mixed and stored in the dark. The absorbance at 410 nm was read within 1 h of acidification. The absorbance at 410 nm was linear with concentration to at least 1.1 absorbance units.

Determination of Vesicle Size Distribution. The homogeneity of the LUV population was determined by column chromatography on Sepharose CL-2B at 4 °C. An upward flowing Pharmacia K16 column (1.6 × 25 cm) was equilibrated at 4 °C with 100 mM KCl. The vesicle dispersions (1–2 mL) were loaded onto the column by a three-way valve and eluted at 10 mL/h.

Nuclear Magnetic Resonance (NMR). The ^{31}P NMR measurements were performed at 24.15 MHz by using a JEOL FX60Q Fourier transform spectrometer. All spectra contained 1K data points after Fourier transformation. The spectra were obtained at 24 °C by using continuous ^1H noise decoupling, a 2000-Hz sweep width, 0.51-s acquisition time, 3.5-s delay between acquisitions, and a 90° pulse of 16 μs. Between 1000 and 2000 scans were accumulated.

Electron Microscopy. Samples at 21 °C were placed on gold planchets and rapidly frozen by plunging into liquid Freon. Fracturing, etching, and shadowing were performed in a Balzers BAF 300 freeze–etch apparatus under a vacuum of 1×10^{-6} mbar. Fracturing was done at −100 °C followed by etching by sublimation for 30 s. Platinum was shadowed at a 45° angle to a depth of 20 Å. A 250-Å carbon film was then applied to the replica. The original sample was cleaved from the replicas by floating on Clorox bleach for 24 h.

Results and Discussion

Before the influence of the radius of curvature on the transbilayer phospholipid distribution could be examined, well-defined populations of large unilamellar vesicles had to be prepared. We have found that such vesicles can be obtained by modifying the ethanol injection procedure described by Batzri & Korn (1973). In their original report, they portrayed the rapid ethanol injection technique as an alternate method for the formation of SUV. Egg PC vesicles made by this method were characterized by gel chromatography, negative-stain electron microscopy, and trapped volume and judged to be identical with sonicated vesicles. Kremer et al. (1977) altered the original procedure by slowly injecting the ethanolic solution into buffer. As a consequence, the average radius of dimyristoyl- and dipalmitoylphosphatidylcholine dispersions, determined by light scattering techniques, was found to increase linearly from 150 to 500 Å as the PC concentration in the ethanol solution increased. The heterogeneity of these dispersions was assessed by using the Zimm plot Z parameter. However, this parameter cannot be readily interpreted in terms of a vesicle radius distribution, particularly for unfractionated dispersions.

We have prepared large vesicles by the slow injection procedure but have handled them in such a way as to obtain

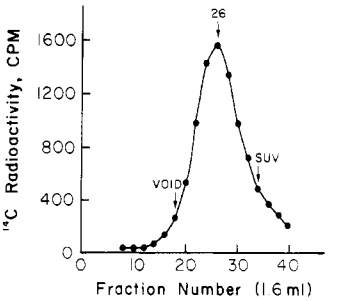

FIGURE 1: Elution profile of 0.50 mol fraction egg PE vesicles centrifuged for 38 min as described in the text. VOID, the excluded volume; SUV, the SUV peak elution volume on a Sepharose CL-2B column. The ordinate indicates counts per minute from the egg phosphatidyl[*N-methyl*-^{14}C]choline incorporated into the vesicles (0.01 mol %).

well-defined, reproducible populations. Basically, the procedure was to remove the ethanol, concentrate the vesicles, and fractionate them by centrifugation (Barenholz et al., 1977). They were characterized by gel chromatography, electron microscopy, and NMR.

In order to obtain the higher concentrations needed for further studies, larger amounts of the ethanolic solution were injected. The final aqueous solution contained as much as 30% ethanol. Although the ethanol was normally removed immediately after vesicle formation, vesicles incubated overnight in the presence of ethanol generated similar gel chromatography profiles as vesicles from the same sample which were stored under identical conditions in the absence of ethanol (data not shown). Radiolabeled ethanol, included in some experiments, indicated that a single pass down a Sephadex G-50 gel column quantitatively removed the ethanol (less than 1 mol of ethanol/10^8 mol of phospholipid remained). This was found to be a more effective procedure than dialysis.

Sepharose CL-2B gel chromatography was used to characterize the approximate size and heterogeneity of slow injection dispersions. Unfractionated dispersions generated very heterogeneous gel distributions, including considerable material in the void volume (data not shown). Upon fractionation by centrifugation, a considerably more homogeneous vesicle population was obtained, as shown in Figure 1. The same average diameter and range of vesicle sizes were present in freeze–etch electron micrographs. A typical micrograph is shown in Figure 2. The diameters of vesicles, uncorrected for variable fracture planes, appear to range from 600 to 800 Å.

Before the transbilayer distribution of phospholipids was assessed, it was necessary to establish that the vesicles were unilamellar. Electron microscopy has been used for this purpose, and we find, from studying micrographs like Figure 2, no evidence for smaller vesicles trapped within larger vesicles. Oligolamellar vesicles, structures containing a few concentric bilayers spaced at the characteristic multibilayer distance, were also not apparent, but further substantiation of these observations was required. A parameter that is quite sensitive to the presence of more than one lamellae per vesicle, particularly for large radius vesicles, is the ratio of the number of molecules exposed to the external medium to the number that is not. This ratio can be measured by ^{31}P nuclear magnetic resonance, with and without a broadening reagent, Mn^{2+}, added to the fractionated vesicles (Bergelson, 1978). For unilamellar vesicles with radii larger than about 400 Å, this ratio is rather insensitive to radius changes, approaching a value of 1.0 asymptotically. If more than one lamella is present, the ratio will be less than 1.0. It can be shown that if 10% of the phospholipid resides in such structures the ratio

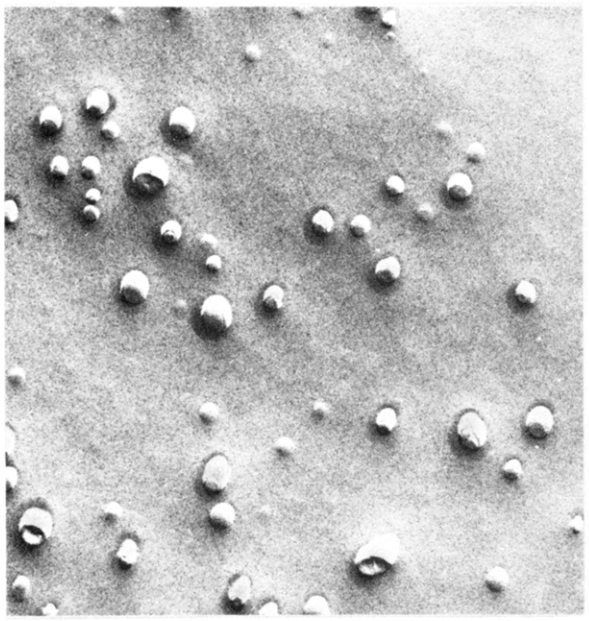

FIGURE 2: Freeze-etched electron micrograph of a fractionated vesicle preparation obtained by the slow ethanol injection method described under Materials and Methods.

Table I: Chromatography of Unilamellar Vesicles

	phospholipid concn in ethanol (mM)	normalized elution volumes (mL)	half-width at half-height (mL)
LUV Chromatographed on Sepharose CL-2B			
egg PC	35	43	13
egg PE–egg PC	10	43	13
dimyristoyl-PC	10	32	13
dipalmitoyl-PC	10	void volume	
SUV Chromatographed on Sepharose CL-4B			
rapid ethanol injection egg PE–egg PC		66	4
sonicated egg PE–egg PC	35	66	

will decrease by about 0.2. The ratio for the fractionated PE/PC vesicles made by the slow injection method was 1.0 ± 0.1. This indicates, in combination with the gel chromatography and electron microscopy results, that the vesicles are large and essentially unilamellar. An important control in measuring this ratio, particularly for large vesicles, is to establish that the broadening reagent does not permeate the vesicle bilayer. This was accomplished by rerunning the ^{31}P NMR spectrum in the presence of Mn^{2+}. For the PC/PE vesicles, the signal from the internal phospholipid did not decrease over a 24-h period, and the original spectral integral could be restored upon addition of sufficient amounts of EDTA. Thus, the vesicles are impermeable to Mn^{2+}.

Since the purpose of this study was to prepare large vesicles, we did not investigate extensively the variables involved in making vesicles of different average radius. However, some preliminary results are given in Table I. At the same phospholipid concentration in ethanol, the average vesicle size for unfractionated preparations increases along the series egg PC, dimyristoylphosphatidylcholine, and dipalmitoylphosphatidylcholine. This is in contrast to the results obtained by Kremer et al. (1977) for dimyristoyl- and dipalmitoylphosphatidylcholine. As the total phospholipid concentration in ethanol increases in any of these systems, so does the average

Table II: Distribution of Egg PE in LUV and SUV

mol % PE	outer surface PE/total PE: ethanol injection LUV	ethanol injection SUV	sonicated[a] SUV
10	0.52 ± 0.01	0.72 ± 0.02	0.70
30	0.49 ± 0.01	0.59 ± 0.02	0.60
50	0.46 ± 0.01	0.54 ± 0.02	0.56

[a] Litman (1974).

vesicular size [unpublished results and Kremer et al. (1977)]. The percentage recovery of vesicles after centrifugation varied with lipid composition and concentration. Thus, the centrifugation step is crucial for the generation of reproducible vesicle populations. It should also be noted that rapid injection of the ethanol solution produces much smaller vesicles than one obtains by slowly injecting the same solution (Table I).

In order to explain these results, it is necessary to speculate about the mechanisms of vesicle formation. As the molar ratio of ethanol to water is decreased in egg PC–ethanol–water dispersions under near equilibrium conditions, the phosphatidylcholine molecules which are initially present as monomers in the ethanolic solution (Kellaway & Saunders, 1970) pass through at least one mixed micellar state before a highly turbid species, presumably lamellar, is formed above 60% water (Tinker & Saunders, 1968; Aarts et al., 1977). Since the slow injection vesicles prepared in this study are apparently stable in solutions containing up to 30% ethanol, we would tentatively identify these vesicles with the high turbidity species of the near equilibrium studies. This is the simplest model. Events, like the formation of larger lamellar species which then break up to form smaller species, cannot be ruled out.

Self-assembly of the injection vesicles appears to occur after 60% of the ethanol in the droplet is replaced by water. This process probably occurs more rapidly during slow injection. Hence, the larger vesicles may represent "trapped" species because the entropically favored smaller vesicles (Israelachvili et al., 1977) do not have time to form. This is supported by the effect of adding ethanol to the medium before injection, which tends to produce smaller vesicles (Kremer et al., 1977).

The increase in vesicle radii with lipid concentration in ethanol can be easily explained on the basis of a dilution model. The effect of fatty acid chain length and the presence of double bonds on the vesicle radius (Table I) is more difficult to rationalize. Presumably, the solubility of PC in ethanol and the mole percent water at which bilayers assemble upon ethanol replacement will decrease as the chain length increases and/or double bonds are removed, so a kinetic effect is possible.

The distribution of PE in large egg PE–egg PC vesicles of this type was determined by TNBS labeling and compared with the PE distribution in SUV of the same phospholipid composition. Large unilamellar vesicles containing 10, 30, and 50 mol % PE were prepared as described, and aliquots of each were assayed after centrifugation. The results, shown in Table II, reveal that PE is equally distributed between the two monolayers over a wide composition range. The downward trend in the ratio of the outer surface PE to the total PE with increasing mole percent PE falls within the limits of accuracy of the labeling procedure and is probably not significant. Regardless of composition, the vesicles displayed similar elution profiles on a Sepharose CL-2B column. The peak eluted at the same volume, and the half-widths at half-height were similar, indicating that within the resolution of the column the size and homogeneity of the various vesicle populations were the same (Table I). Small unilamellar PE–PC vesicles were

prepared by rapid ethanol injection or probe sonication. The PE distribution was similar for vesicles made by either method (Table II) and agreed with the published results of Litman (1974). PE was preferentially localized in the outer monolayer at low PE concentrations and in the inner monolayer at higher PE concentrations. The elution profiles on Sepharose CL-4B were similar (Table I), indicating that small vesicles made by ethanol injection or probe sonication are probably structurally equivalent. This suggests that the observed phospholipid asymmetry in small unilamellar vesicles made by these methods (one dispersive, the other aggregative) is a consequence of the highly curved bilayer structure rather than the mechanism of vesicle formation.

Further studies on the PE–PC SUV system suggest the various ways in which this geometric parameter can exert its influence. Litman (1975) showed that the surface distribution of the fatty acid side chains of egg PE was random and independent of the mole fraction of PE in egg PE–egg PC SUV. It was concluded that head-group packing requirements dictated the surface distribution of egg PE and egg PC in this system. More recent investigations by Lentz & Litman (1978) on dimyristoylphosphatidylcholine–dimyristoylphosphatidylethanolamine sonicated vesicles demonstrated the influence acyl side chains can have on phospholipid asymmetry. In this system, a disproportionate amount of PE is found in the outer monolayer at any mole percent PE. These results confirmed an earlier observation by Litman (1975) that saturated phosphatidylethanolamines distribute in the outer monolayer more readily than egg PE at the same mole fraction.

Israelachvili et al. (1977) proposed a simple theory to rationalize these observations. Amphipathic molecules are thought to form micelles or bilayers by balancing two opposing forces (Tanford, 1980). The hydrophobic interactions between the hydrocarbon tails which induce the molecules to aggregate are opposed by the requirement of the hydrophilic head groups to remain in contact with the aqueous medium. A compromise ensues that minimizes the total free energy per lipid molecule. However, before the structure can be described, geometric considerations must be introduced. An expression that relates the optimal hydrocarbon–water interfacial area, the hydrocarbon chain volume, and the hydrocarbon chain thickness to the radius of curvature must be formulated for each component. Thus, the structural characteristics of small vesicles are determined in part by the different geometric packing constraints of its components. When the radius becomes much greater than the hydrocarbon chain length, the packing constraints become less important. This suggests a reason why phospholipid asymmetry is absent in large vesicles not subject to the same geometric constraints as SUV.

In summary, the phospholipid asymmetry observed in egg PE–egg PC small unilamellar vesicles is absent in large unilamellar vesicles having the same phospholipid composition. The only difference in these structures is the curvature of the bilayer forming the vesicles. Although phospholipid asymmetry may be generated by highly curved surfaces in some biological membranes, the asymmetry observed in the generally planar biological membrane systems must result from the interaction of phospholipids with other membrane constituents or be a result of the transmembrane potential (McLaughlin & Harary, 1974) or of the mechanisms of membrane biogenesis. Thus, model system investigations will be most fruitful if they are pursued in compositionally asymmetric bilayer systems unbiased by small radii of curvature.

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