Determination of Phosphatidylglycerol Asymmetry in Small, Unilamellar Vesicles by Chemical Modification†

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ABSTRACT: We report the adaptation of a simple, precise chemical assay for vicinal hydroxyl groups to the determination of phosphatidylglycerol transbilayer distribution in small, unilamellar vesicles. Conditions are described under which the chemical procedure reveals only phosphatidylglycerol molecules exposed on the surface of intact phospholipid vesicles. Other assay conditions reveal all the phosphatidylglycerol in the vesicle. Vesicle size distributions have been determined by gel permeation chromatography. These and the phosphatidylglycerol distribution data have been used to estimate the lipid asymmetry in dipentadecanoylphosphatidylglycerol–dimyristoylphosphatidylcholine vesicles. The result obtained for 50 mol % phosphatidylglycerol is similar to earlier nuclear magnetic resonance estimates of the asymmetry in egg phosphatidylglycerol–egg phosphatidylcholine vesicles [Michaelson, D. M., Horwitz, A. F., & Klein, M. P. (1973) *Biochemistry 12*, 2637–2645]. The observed increase in asymmetry at low phosphatidylglycerol content is consistent with interpretations of cationic dye binding data [Massari, S., Pascolini, D., & Gradenigo, G. (1978) *Biochemistry 17*, 4465–4469]. The data are inconsistent with a published theoretical prediction of phosphatidylglycerol asymmetry based solely on electrostatic effects [Israelachvili, J. N. (1973) *Biochim. Biophys. Acta 323*, 659–663]. Rather, the variation of phosphatidylglycerol distribution with vesicle composition is interpreted in terms of both electrostatic effects and different phospholipid packing in the inner and outer leaflets of a small vesicle bilayer.

The effects of charged phospholipids on membrane phase structure have become increasingly of interest [reviewed by Lee (1977)] due to their possible involvement in membrane fusion (Papahadjopoulos et al., 1977; Portis et al., 1979). A popular system for such studies has been phosphatidylglycerol–phosphatidylcholine mixtures (Findley & Barton, 1978; Dombrose et al., 1979), mainly because of the relative ease of phosphatidylglycerol synthesis (Dawson, 1967). Definition of the phase structure of phosphatidylglycerol (PG)[1]–phosphatidylcholine (PC) small, unilamellar vesicles must involve determination of phospholipid transbilayer distribution (Lentz & Litman, 1978). This is especially true when these vesicles are to be used to quantitate the surface binding of such ligands as multivalent metal ions or extrinsic membrane proteins. The transbilayer distribution of phosphatidylglycerol has been estimated previously by nuclear magnetic resonance techniques (Michaelson et al., 1973) in sonicated vesicles prepared from an equimolar mixture of egg yolk derived PC and PG. It was qualitatively reported that PG disproportionated to the outer surface of these vesicles. A theoretical treatment has attributed this result to the contribution of electrostatics to the free energy of vesicle formation (Israelachvili, 1973). This theory cannot be tested until an accurate definition of lipid asymmetry is available through combined lipid distribution and vesicle size determinations. Since the transbilayer distribution of phosphatidylethanolamine (PE) in small, unilamellar vesicles has been shown to vary considerably with PE/PC ratio (Litman, 1973; Lentz & Litman, 1978), it is clearly important to define the distribution of charged lipids as a function of vesicle composition. This will be difficult to accomplish by nuclear magnetic resonance measurements since these require large quantities of lipid. Recently, a cation dye binding procedure has been used to obtain estimates of the distribution of charged phospholipids (Massari et al., 1978). The results obtained were qualitatively reasonable, but uncertainties in the binding technique and in the nature of the vesicle preparation made quantitative interpretation difficult. Clearly, more measurements are required to test models that propose to explain the disproportionation of charged lipids in highly curved membranes. Therefore, we report here the adaptation of a simple chemical assay for vicinal hydroxyl groups to the precise determination of phosphatidylglycerol transbilayer distribution in small, unilamellar vesicles.

Materials and Methods

Phospholipids. The synthesis and purification of 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC) was as previously described (Lentz et al., 1976). The DMPC stock was radioactively labeled with [1-^{14}C]DMPC (Applied Science, lot B0141; 1 mmol/mol of synthetic DMPC). For column chromatographic determinations of vesicle size, it was necessary to incorporate a small amount of foreign tracer lipid ([U-^{14}C]phosphatidylcholine, Applied Science) at a level of less than 0.03 mol % contamination. The sodium salt of 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol ($DC_{15}PG$) was specially prepared by Avanti Biochemicals (Birmingham, AL) according to the procedures of Dawson (1967). The commercial material showed 2 to 3% nonphosphate impurities which were removed by two acetone–chloroform precipitations (Lentz et al., 1976). Both lipids were judged better than 99% pure by thin-layer chromatography on Quantum (Fairfield, NJ) Q5W plates loaded with 1 to 2 μmol of lipid (Lentz et al., 1976) and developed with chloroform–methanol–water (65:25:4) or chloroform–methanol–water–acetic acid (65:25:2.5:2.5). DMPC was stored in spectral grade chloro-

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[1] Abbreviations used: DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; $DC_{15}PG$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; CF, 6-carboxyfluorescein; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine.

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form, and $DC_{15}PG$ was stored in 1:1 spectral chloroform–glass-distilled methanol at 5 μmol/mL concentration under an argon atmosphere at −70 °C. Lipid concentration of the stock solutions was established by phosphate determination (Chen et al., 1956). Vesicle $DC_{15}PG$ content was determined as described below, while DMPC content was established by scintillation counting.

Small, Unilamellar Vesicles. Mixtures of DMPC with $DC_{15}PG$ were prepared in chloroform–methanol and dried in a thin film onto the wall of a glass ampule under a stream of argon. Phospholipids to be used in asymmetry assays were dispersed in buffer (100 mM NaCl and 10 mM sodium cacodylate, pH 7.1) at 42 °C (above the phase transitions of both components) to a final concentration of 0.2–1 mM in total phospholipid. Vesicles used for gel chromatography experiments were prepared in a different buffer: 100 mM NaCl, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), and 0.02% sodium azide, pH 7.4. Phospholipid suspensions were sonicated directly in the ampule at 42 °C by using a Heat Systems W350 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) equipped with a Heat Systems Cup-Horn (Barrow & Lentz, 1980). The traditional immersed titanium tip sonication technique (Huang, 1969) led to inconsistent estimates of lipid asymmetry in these anionic–neutral lipid mixtures. Sonicated preparations were centrifuged (Barenholz et al., 1977) in a Beckman Ti 50 rotor at 40–50 °C at 186000*g* for sufficient time to yield a well-defined small vesicle population (see Results). Region III vesicles (Barenholz et al., 1977) were stored at 42 °C under argon until used (usually less than 12 h).

Determination of Phosphatidylglycerol Transbilayer Distribution. An adaptation of the vicinal hydroxyl group assay of Yang et al. (1967) was used to determine exposed PG under conditions in which vesicles remained intact. Total PG was determined with vesicles disrupted by sodium dodecyl sulfate ($NaDodSO_4$). All assays were carried out at 42 °C so as to be above the phase transition temperatures of both lipids involved (23.7 °C, DMPC; 33.5 °C, $DC_{15}PG$). The *exposed phosphatidylglycerol* was determined as follows. A vesicle aliquot containing 0.01–0.10 μmol of exposed PG was placed in a culture tube (Teflon-capped Pyrex no. 9826) and diluted to 0.9 mL with buffer. Many common buffers interfered with this assay, leading us to the adoption of the cacodylate buffer system. Each sample was adjusted to pH 5.5 by the addition of 0.1 mL of 1 M sodium acetate–acetic acid with thorough mixing. Next, 0.05 mL of 0.1 M sodium periodate was added with mixing and the oxidation of exposed PG was allowed to proceed for 9–22 min. After appropriate time intervals, the reaction was terminated by the addition of 0.1 mL of 5% (w/v) sodium bisulfite solution, containing 0.05 M $NaDodSO_4$ for the purpose of disrupting the vesicles. The determination of *total phosphatidylglycerol* was similar except that the sodium acetate–acetic acid buffer contained 0.05 M $NaDodSO_4$, which was omitted from the sodium bisulfite solution.

Once the oxidation of the vicinal hydroxyl groups of PG was accomplished, determination of the "exposed" and "total" PG was made by the same procedure. To each culture tube was added 2 mL of 0.275 mM chromotropic acid (2,5-dihydroxynaphthalene-2,7-disulfonic acid; Sigma Chemical) in 27 N sulfuric acid. The culture tubes were thoroughly vortexed and then heated in a boiling water bath for 35 min. After the mixture was cooled to room temperature, 0.5 mL of 12% (w/v) thiourea solution was added with mixing. The resulting purple solution was centrifuged at subambient temperature. This removed bubbles and separated an oily residue that otherwise interfered with spectrophotometric determination of the assay products. The transparent bottom layer was removed from each culture tube with care not to introduce bubbles or disturb the oily top layer. The optical density of this solution was read in a Hitachi 100-20 spectrophotometer at 570 nm and related by standard curves to the PG present. Standard curves were determined by assay of aliquots of a $DC_{15}PG$ standard solution by the total procedure. Blanks were obtained by performing the total assay in the absence of lipid.

Carboxyfluorescein Permeability. 6-Carboxyfluorescein (CF) was purchased from Molecular Probes (Plano, TX) and used without further purification. $DC_{15}PG$–DMPC small, unilamellar vesicles were prepared in cacodylate buffer containing 0.1 M CF. These were separated from external CF by passage through a Sephadex G-50 column. The resultant vesicles, containing trapped CF, were stored at 4 °C before slow warming (20 °C/h) to 40 °C for assay of external $DC_{15}PG$. The release of CF during the assay procedure was monitored through the increased fluorescence (Blumenthal et al., 1977) detected at 515 nm after excitation at 491 nm in an SLM Instruments (Urbana, IL) 4800 spectrofluorometer. Increased fluorescence results from relaxation of self-quenching effects observed at high concentrations of this fluorophore.

Vesicle Size Distribution Determination. Vesicle populations were characterized by chromatography on Sepharose CL 4B at 45 °C. A 1.2-cm water-jacketted column (Glenco Scientific) was packed to a bed height of 15 cm and equilibrated in 0.01 M Tes, 0.1 M NaCl, and 0.02% sodium azide buffer (pH 7.4). The vesicle sample was loaded onto the column by means of a four-way valve (Rheodyne Type 50, Berkeley, CA) in a total volume of 0.1 mL. The flow rate through the column was maintained at 2 mL/h by means of a Sage 352 nonpulsating infusion pump. One-drop fractions (~50 μL) were collected directly into scintillation vials, scintillated, and counted as a record of column elution.

Results

Development of the Assay. The reaction procedure for the PG vicinal hydroxyl assay involved two different steps. The first step of the assay utilized the well-known periodate oxidation of a 1,2-diol to formaldehyde and a residual aldehyde (Allinger et al., 1971). The second step in the assay was a colorimetric assay for formaldehyde (Feigl, 1966) involving condensation of two molecules of chromotropic acid with each molecule of formaldehyde. An important point is that only the first step took place at the surface of the intact membrane. This involved a mild oxidation and was terminated by the addition of excess sodium bisulfite, a reducing agent. The second step involved severe conditions and was carried out with disrupted vesicles. However, formaldehyde was detected at this point only if produced from PG exposed during the first step. The complete assay was found to be linear in the presence of $NaDodSO_4$ from 0 to 0.22 μmol of PG with a proportionality constant of 4.81 ± 0.07 OD/(μmol cm).

The success of any chemical modification procedure for determining membrane phospholipid asymmetry depends upon the inaccessibility of one side of the membrane to the modifying reagent. This requires that the membrane remain intact and impermeable during the incubation time required for the covalent modification. To demonstrate this in our system, it is necessary to show that the amount of reacted PG remains constant over an extended time in both intact and disrupted vesicle samples. Figure 1 shows the time dependence of the exposed and total PG oxidation in 1:1 DMPC–$DC_{15}PG$ vesicles. The exposed and total PG pools are defined operationally by the different assay conditions described under Materials

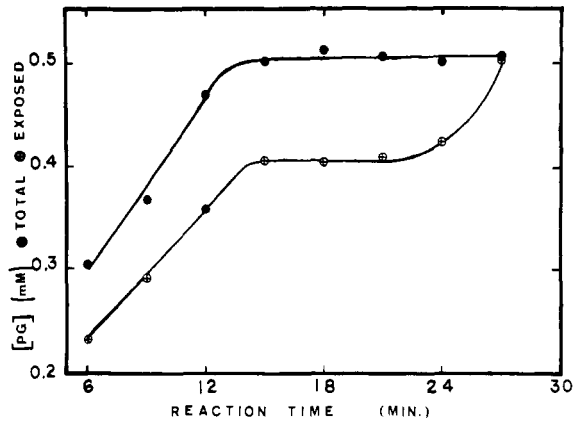

FIGURE 1: Time course of the total (●) and exposed (⊕) PG assay as a function of the length of the oxidation step for a 0.49 mol fraction $DC_{15}PG$ sample of total lipid concentration 1.16 mM.

and Methods. The measured PG in both pools remained essentially constant in the range of 15–23 min total reaction time (Figure 1). Essentially similar results were obtained for vesicles of low (15 mol %) and high (80 mol %) $DC_{15}PG$ content. When the assay was allowed to run for more than 22 to 23 min, the amount of exposed PG rose rapidly to the total value (Figure 1). This suggests a rapid increase of vesicle permeability to periodate after an initial period of impermeability. Performing the oxidation of vicinal hydroxyl groups at higher pH (e.g., 7.0) shortened the period of impermeability.

While the results in Figure 1 are consistent with the existence of a permeability barrier up to 22 to 23 min, there are other possible explanations for these complex kinetics. In order to directly test our interpretation, we have monitored vesicle permeability during the course of periodate oxidation by measuring the release of trapped CF (Blumenthal et al., 1977). As previously reported, the leakage of CF from intact vesicles at high temperature and acidic pH was observed to be appreciable (Szoka et al., 1979). As shown in Figure 2, the leakage rates in control (periodate-free) and oxidized (periodate-exposed) 17 mol % PG vesicles were essentially identical for roughly 20 min following the addition of periodate. However, beyond 20 min the remaining CF was much more rapidly lost from periodate-exposed vesicles than from unexposed vesicles. Very similar results were obtained in studies of 1:1 $DC_{15}PG$–DMPC vesicles (see caption to Figure 2). These results demonstrate a dramatic increase of vesicle permeability after roughly 20–22 min of exposure to periodate oxidation and, therefore, support our interpretation of Figure 1.

Characterization of Vesicle Populations. It is well-known that the fraction of total vesicle phospholipid exposed to the external aqueous milieu is a sensitive function of vesicle size (Sheetz & Chan, 1972). This fraction may vary from 5% for large, multilamellar vesicles (Schwartz & McConnell, 1978) to 70% for phosphatidylcholine small, unilamellar vesicles isolated by ultracentrifugation (Barenholz et al., 1977). Thus, we must demonstrate that our procedures isolated a reasonably uniform small vesicle population in which the exposure of PG to our assay procedure did not vary with further fractionation. Figure 3 illustrates the effect of increasing centrifugation times on the recovery of PG (A) and on the exposure of PG to covalent modification (B). As reported for egg PC–PE vesicles (Barenholz et al., 1977), loss of lipid leveled off after 90 min of centrifugation at 168000*g*, indicating a highly uniform vesicle population. However, the ratio of exposed to total PG had reached a limiting value after only 30 min of centrifugation, suggesting that a population sufficiently homogeneous

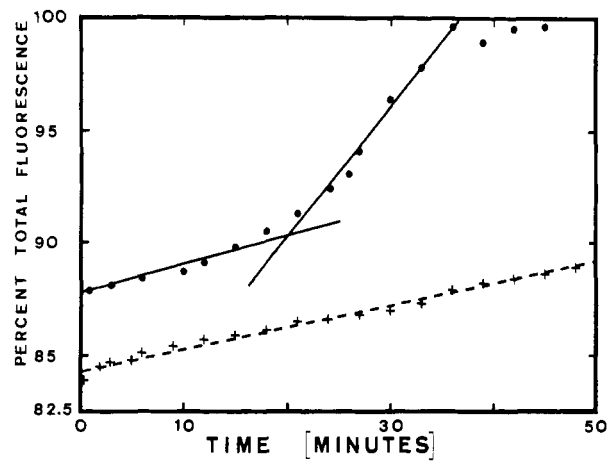

FIGURE 2: Leakage of CF from 17:83 $DC_{15}PG$–DMPC vesicles. The observed fluorescence is plotted as the percent of fluorescence observed when vesicles were disrupted by addition of $NaDodSO_4$. Linear increases in fluorescence in the presence (●) and absence (+) of periodate yielded first-order rate constants (obtained by linear regression analysis) for CF leakage of 2.8 ± 0.3 and 2.8 ± 0.1 ns^{-1}, respectively. The rate constant for CF leakage from periodate-treated vesicles increased dramatically after 20 min to 16.7 ± 1 ns^{-1}. Similar results were obtained with 1:1 $DC_{15}PG$–DMPC vesicles except that CF leakage rates were 8.9 ± 0.8 and 8.7 ± 0.7 ns^{-1} in the presence and absence of periodate at times less than 22 min of exposure to periodate and 39.7 ● 7 ns^{-1} after 22 min of exposure to periodate.

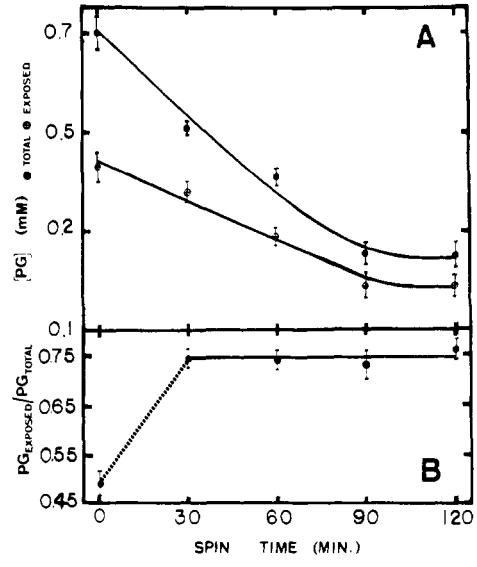

FIGURE 3: Effect of fractionation by centrifugation on the measured asymmetric distribution of PG in sonicated vesicles. On the abscissa is recorded the centrifugation time at 168000*g* in a Beckman Ti 50 rotor. The total (●) and exposed (⊕) PG assays for a 0.67 mol fraction $DC_{15}PG$ vesicle sample are plotted on the ordinate in (A), while the ratio of these quantities is plotted in (B). Error bars represent plus or minus one standard deviation.

for our studies could be obtained in less than 90 min with less loss of lipid. Similar results were obtained at low (17%) and high (67%) PG content in $DC_{15}PG$–DMPC vesicles. As a consequence, a compromise centrifugation time of 60 min was used in all vesicle preparations.

The vesicle size distribution was established for each vesicle composition studied by analytical gel permeation chromatography in order to demonstrate the extent to which PG exposure reflects lipid disproportionation across the bilayer. A typical elution profile is shown in Figure 4. Several points can be made about this and other observed profiles. First, the mass-weighted peak position was equal to the unweighted peak position, indicating an unskewed distribution of vesicle sizes.

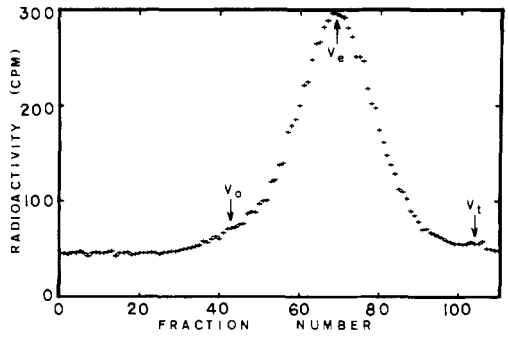

FIGURE 4: Elution profile of 0.15 mol fraction $DC_{15}PG$ vesicles centrifuged for 60 min as described in the text. V_0, the excluded volume; V_t, the total volume of the Sepharose CL 4B column; V_e, the mass-weighed peak of the elution profile. The ordinate indicates counts per minute from the [1-^{14}C]DMPC incorporated into the vesicles.

Second, the vesicle peak was well removed from both the excluded (V_0) and total (V_t) volumes. Thus, the vesicles partitioned well into the gel and their elution position should have accurately reflected vesicle size. For example, the column was able to resolve the difference between egg PC vesicles (peak fraction 69 ± 1; reported mass-distributed peak size by photon correlation light scattering, 100-Å radius; B. J. Litman, unpublished results) and dioleoylphosphatidylcholine vesicles (peak fraction 63 ± 1; reported, 110-Å radius; B. J. Litman, unpublished results). Third, preparations centrifuged for 60 or 90 min did not show the void volume peak typically associated with the larger vesicles found in unfractionated preparations (Huang, 1969; Barenholz et al., 1977). Finally, the vesicle peak positions for all DMPC/$DC_{15}PG$ ratios fell within a narrow range (fraction 68 ± 2) and showed no trend with composition. Using egg phosphatidylcholine and dioleoylphosphatidylcholine as standards, we were able to assign a radius of 100 ± 4 Å to all $DC_{15}PG$–DMPC mixtures studied here.

Extent of Phosphatidylglycerol Exposure. The fraction of PG exposed in DMPC–$DC_{15}PG$ vesicles is plotted in Figure 5 as a function of vesicle composition. Each point shown reflects the average of several measurements made at different reaction times as illustrated in Figure 1. Sonication of lipid suspensions containing large proportions of $DC_{15}PG$ was very difficult, and the yield of small vesicles obtained after centrifugation was quite low for these samples. Attempts to prepare and characterize pure $DC_{15}PG$ vesicles yielded an exposed/total ratio of 0.5, a value inconsistent with those obtained in mixed vesicles. Also, phase transitions in these preparations were history dependent (B. R. Lentz and D. R. Alford, unpublished results). For these reasons, no data are shown for pure $DC_{15}PG$ small vesicles.

Discussion

The simplest interpretation of our results is that the exposed population represented those PG molecules located in the outer monolayer of the vesicle bilayer. This interpretation is supported by the observed constant ratio of exposed to total PG over a significant range of assay time (Figure 1) and by the dramatic increase in CF leakage after 20–22 min of periodate oxidation (Figure 2). Apparently, the membrane bilayer maintained a permeability barrier to the sodium periodate oxidizing agent for a period of time after the bilayer was covalently modified. Conversely, when the vesicles were treated with $NaDodSO_4$, we presume that all the PG became available for oxidation by the sodium periodate. This conclusion is supported by several observations. First, the color developed by the total assay remained constant for the longest

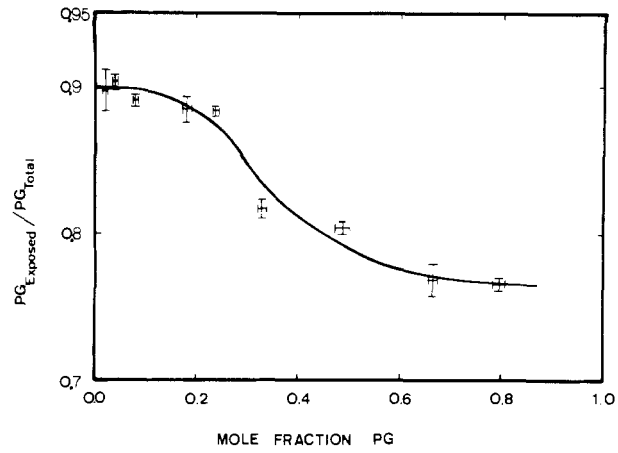

FIGURE 5: Ratio of exposed to total phosphatidylglycerol in small unilamellar vesicles as a function of vesicle composition. Error bars indicate plus or minus one standard deviation.

assay times examined (up to 30 min). Second, performance of the assay in the presence of varying amounts of $NaDodSO_4$ did not change the derived extinction coefficient. Third, addition of the assay quantity of $NaDodSO_4$ caused total release of trapped CF. Finally, vesicle compositions derived from scintillation counting of DMPC and assays for $DC_{15}PG$ always agreed within 2% with the compositions expected on the basis of the known stock solution concentrations of both lipids, as determined by phosphate analysis. In summary, it is most likely that the procedure presented here reveals the ratio of outer surface to total PG molecules and thereby provides a measure of phospholipid distribution in small, unilamellar vesicles.

The data in Figure 5 clearly show a dramatic change with composition in the exposure of PG on the surface of DMPC–$DC_{15}PG$ small, unilamellar vesicles. In order to demonstrate that change in exposure reflects a change in lipid distribution between the vesicle monolayers, we must consider the variation of vesicle geometry with composition. Our gel chromatography results indicate that $DC_{15}PG$–DMPC vesicles were roughly the same size as egg PC vesicles and that this size did not vary significantly with composition. We conclude that the changes in lipid distribution depicted in Figure 5 are reflective of changes in phospholipid asymmetry with vesicle composition. A more quantititative statement requires an estimate of the bilayer thickness or, more directly, the ratio of total outer surface to inner surface phospholipid. Since DMPC bilayers above their phase transition have about the same thickness (Janiak et al., 1976) as egg PC bilayers (Wilkins et al., 1971), we assume that the measured outer/inner surface ratio for egg PC small vesicles (2.2; Barenholz et al., 1977) is appropriate to our vesicles. This reasonable assumption leads to the mole fractions of $DC_{15}PG$ in the outer ($X_{DC_{15}PG}^{out}$) and inner ($X_{DC_{15}PG}^{in}$) monolayers, which are recorded in Table I. The values in Table I make clear the extent of asymmetry at low $DC_{15}PG$ content and the dramatic reduction in this asymmetry with increasing phosphatidylglycerol content.

It is interesting to compare our results with others reported for PC–PG vesicles. At 50 mol % PG, our ratio for the external to total PG (0.79, from Figure 5) agrees well with a value of 0.8 derived from the proton magnetic resonance data of Michaelson et al. (1973), who studied a mixture of unsaturated lipids derived from egg yolk. A recent report (Massari et al., 1978) has inferred an external/total PG ratio from studies of the binding of acridine orange dye to sonicated dispersions of egg phosphatidylglycerol–dipalmitoylphosphatidylcholine. The ratios reported by Massari et al.

Table I: Mole Fraction of $DC_{15}PG$ in the Outer and Inner Monolayers of DMPC-$DC_{15}PG$ Vesicles

mole fraction of $DC_{15}PG$	$X_{DC_{15}PG}^{out}$	$X_{DC_{15}PG}^{in}$	$X_{DC_{15}PG}^{out}/X_{DC_{15}PG}^{in}$
0.021 ± 0.002	0.027 ± 0.003	0.007 ± 0.002	3.9 ± 1.3
0.042 ± 0.002	0.055 ± 0.003	0.013 ± 0.002	4.2 ± 0.8
0.080 ± 0.004	0.103 ± 0.006	0.028 ± 0.002	3.7 ± 0.5
0.181 ± 0.009	0.232 ± 0.014	0.067 ± 0.007	3.5 ± 0.7
0.238 ± 0.005	0.305 ± 0.007	0.084 ± 0.004	3.4 ± 0.2
0.330 ± 0.007	0.391 ± 0.011	0.195 ± 0.011	2.0 ± 0.2
0.487 ± 0.015	0.57 ± 0.02	0.31 ± 0.02	1.8 ± 0.2
0.665 ± 0.008	0.74 ± 0.02	0.50 ± 0.03	1.5 ± 0.1
0.795 ± 0.013	0.88 ± 0.02	0.60 ± 0.02	1.5 ± 0.1

approach the values obtained here at low phosphatidylglycerol content but show increasingly negative deviation from our results with increasing PG content. These differences may reflect the very different lipid mixtures studied or may result from changes in the binding properties of acridine orange with increasing vesicle surface charge density. Finally, theoretical predictions of the asymmetric phospholipid distribution in highly curved, charged membranes have been based on a simple electrostatic model (Israelachvili, 1973) and agree well with our results at 50 mol % $DC_{15}PG$ (predicted PG_{out}/PG_{in} = 4.2; 4.0 obtained here by multiplying X_{PG}^{out}/X_{PG}^{in} by the ratio of total outer leaflet to inner leaflet lipid, 2.2). However, this theory predicts the same ratio of exposed to total PG at all compositions and is, therefore, in conflict with our observations. This suggests that something other than electrostatic forces must be involved in determining the distribution of PG across highly curved membranes.

The parallel between Figure 5 and similar plots reported for the variation of external to total phosphatidylethanolamine with composition in small, unilamellar vesicles (Litman, 1973; Lentz & Litman, 1978) suggests a common origin for the lipid asymmetry observed in these two systems. The similarity between these two very different lipids (PG and PE) implies that their disproportionation was directed not by their physical properties but rather by the properties of the lattice in which they were situated. It seems reasonable, therefore, to consider these phospholipids as "impurities" in the phosphatidylcholine small vesicle lattice. At low mole fraction, the "impurity" lipids so far examined dramatically disproportionated to the outer surface to the extent that the outer surface concentration became roughly 4 times that of the inner surface [Table I; see also Lentz & Litman (1978)]. At very low mole fraction, it is likely that the contribution of mixing entropy outweighed this lattice effect [e.g., Figure 5 levels off at very low PG content; cf. especially Figure 3 of Lentz & Litman (1978)]. By contrast, the inherent physical properties of the noncholine lipids should have had increased influence on lipid distribution at high "impurity lipid" contents. Thus, PG, because of its charge, disproportionated somewhat to the outer surface at high PG content (i.e., in Figure 5 $PG_{external}/PG_{total}$ approaches 0.78 rather than 0.7 which would be expected for a random distribution). Phosphatidylethanolamine, on the other hand, because of its small head group, favored slightly the inner surface (Litman, 1973). Such an interpretation is consistent with the presumed structural differences between the inner and outer monolayers associated with a highly curved vesicle (Huang & Mason, 1978) and suggests that highly curved regions of biological membranes may contain disproportionate concentrations of such lattice impurities as membrane-associated proteins.

Acknowledgments

We thank Dr. Y. Barenholz and D. Barrow for reading and criticizing the manuscript.

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