Transbilayer Redistribution of Phosphatidylglycerol in Small, Unilamellar Vesicles Induced by Specific Divalent Cations†

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ABSTRACT: We have investigated the effect of low levels of divalent metal ions on the transbilayer distribution of phosphatidylglycerol in small, unilamellar vesicles containing egg phosphatidylglycerol/egg phosphatidylcholine or dipentadecanoylphosphatidylglycerol/dimyristoylphosphatidylcholine mixtures. A chemical assay for phosphatidylglycerol was used to measure the fraction of total phosphatidylglycerol exposed on the exterior vesicle surface in the absence and presence of di- and trivalent metal ions. In addition, ^{1}H NMR spectra were analyzed to obtain independent estimates of phosphatidylcholine transbilayer distribution in the presence and absence of Mn^{2+}. All data were consistent with the hypothesis that Mn^{2+} and Cd^{2+} induce the transbilayer redistribution of lipids in these vesicles. No other metal ions tested (Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Ti^{2+}, Eu^{3+}, and Pr^{3+}) caused redistribution. The rate of lipid redistribution was found to be dependent on the concentrations of lipid and ion in the reaction mixture. Dose–response experiments showed Mn^{2+}/phospholipid ratios as low as 1/10 000 to be sufficient to induce the lipid redistribution after 2.5-h incubation. We have concluded that Mn^{2+} and Cd^{2+} act as catalysts allowing the spontaneous redistribution of phosphatidylglycerol and phosphatidylcholine in small, unilamellar vesicles. Our results also suggest that the transbilayer distribution of lipids in freshly prepared small, unilamellar vesicles may not be an equilibrium distribution. Instead, the transbilayer distribution probably represents a kinetically trapped arrangement characteristic of the procedure used to form the vesicles. Finally, we determined the presumed equilibrium distribution of phosphatidylglycerol in vesicles after incubation with Mn^{2+}. The dependence of this lipid transbilayer distribution on vesicle composition was determined and is discussed in terms of our previously proposed model for the thermodynamic forces driving membrane asymmetry in small, unilamellar vesicles [Lentz, B. R., Alford, D. R., & Dombrose, F. A. (1980) *Biochemistry 19*, 2555–2559].

The distribution of lipids between the two leaflets of several cellular membranes is thought to be asymmetric (Etemadi, 1980). This asymmetry may have functional significance in a variety of membrane-mediated processes such as enzyme function (Etemadi, 1980), enhancement of the rate of blood coagulation by platelet membranes (Shick, 1978; Zwaal, 1978), and cell fusion (Cullis & DeKruijff, 1979). Phospholipids in the model membranes of small, unilamellar vesicles (SUV)[1] have also been found to be asymmetrically disposed between the two membrane leaflets. Asymmetry in these model membranes has been studied fairly extensively to gain insight into the dynamic interactions governing the arrangements of phospholipids in both highly curved and uncurved regions of biological membranes (Litman, 1973; Berden et al., 1975; Lentz & Litman, 1978; Carnie et al., 1979; Barsukov et al., 1980; Lentz et al., 1980; Nordlund et al., 1981). Lipid asymmetry in SUV membranes has been proposed to result from the different packing geometries available in the two monolayers of these highly curved membranes. The most clear picture of curvature-induced asymmetry has emerged for mixtures of phosphatidylethanolamine and phosphatidylcholine, both of which are zwitterionic at neutral pH. Phosphatidylethanolamine has been shown to prefer the outer membrane leaflet at low mole fraction, but to distribute to the inner leaflet or remain equally distributed at higher mole fraction, depending on the acyl chain composition of the lipids (Litman, 1973; Lentz & Litman, 1978). For mixtures of charged and zwitterionic phospholipids, the picture is less clear. Nuclear magnetic resonance (NMR)[1] studies utilizing paramagnetic shift reagents have suggested that mixtures of egg phosphatidylcholine (PC)[1] with both bovine phosphatidylserine and egg phosphatidylglycerol (PG)[1] formed SUV with enhanced charged lipid concentration in the inner membrane leaflet, especially at low charged lipid content (Berden et al., 1975; Barsukov et al., 1980). However, results obtained by a dye-binding method (Massari et al., 1978) or by chemical modification of exposed charged lipids (Lentz et al., 1980; Massari et al., 1978) have led to exactly the opposite conclusion; the charged lipid disproportionated to the outer monolayer especially at low charged lipid content. While uncertainties about vesicle size, method of preparation, and the effects of concentrated shift reagents plagued some of the above-mentioned NMR studies, a very recent publication (Nordlund et al., 1981) carefully controlled these variables in experiments with egg PG/egg PC vesicles. This report concluded that vesicles composed of between 12 and 74 mol % PG showed no asymmetry in the distribution of PG, a conclusion at odds with both our chemical modification experiments (Lentz et al., 1980) utilizing well-defined SUV populations and dye binding experiments from another laboratory (Massari et al., 1978).

In an effort to resolve some of these discrepancies, we investigated several parameters which might account for the different conclusions resulting from our work and the careful NMR studies of Nordlund et al. (1981). This effort has led to a surprising discovery: small amounts of Mn^{2+} and Cd^{2+} added to PG/PC SUV lead to transbilayer redistribution of lipids. We have found that this effect is specific for certain divalent cations and appears to be catalytic in nature. In

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[1] Abbreviations: SUV, small, unilamellar vesicles; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PG, phosphatidylglycerol; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; $DC_{15}PG$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid.

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addition, the transbilayer distribution of PG is sensitive to the sonication procedure used to prepare SUV. Taken together, our results lead to the conclusion that the transbilayer distribution of lipids in SUV need not represent an equilibrium arrangement.

Materials and Methods

Phospholipids and Other Reagents. The synthesis and purification of 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC)[1] and the isolation of chicken egg yolk phosphatidylcholine (egg PC)[1] were as described previously (Lentz et al., 1976). Stocks of DMPC (25 mM) and egg PC (35 mM) were prepared in HPLC-grade, argon-bubbled chloroform and were radiolabeled with [1-^{14}C]DMPC to a level of 3.2 mmol of labeled lipid/mol of unlabeled lipid. [1-^{14}C]DMPC was prepared by the method of Patel et al. (1979) from [1-^{14}C]-myristic acid purchased from Research Products International Corp. (Mount Prospect, IL), batch 1076-178. The sodium salt of 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol ($DC_{15}PG$)[1] was specially prepared by Avanti Biochemicals (Birmingham, AL) according to the procedures of Dawson (1967). The commercial, chromatographically pure material was further purified by extraction with disodium ethylenediaminetetraacetate (Na_2EDTA)[1] solution followed by precipitation from acetone (Lentz et al., 1980, 1982). Phosphatidylcholines were similarly treated. Egg phosphatidylglycerol (egg PG) was prepared by Avanti Biochemicals from egg PC by an entirely analogous procedure. Both phosphatidylglycerols were stocked as their sodium salts in argon-bubbled 1/1 chloroform/methanol (HPLC grades) solution at concentrations of 8–10 mM. All lipids were judged to be better than 98% pure by thin-layer chromatography (Lentz et al., 1976, 1980). Flame-ionization atomic absorption analysis on a Perkin-Elmer 305B atomic absorption spectrometer showed the stock PG solutions to contain less than 2 mol of Ca^{2+}/100 mol of lipid, while the PC stocks contained less than 1 mol of Ca^{2+}/100 mol of lipid. All lipid stocks were stored at −70 °C and layered with argon. Lipid concentrations of the stock solutions were established by phosphate determinations (Chen et al., 1956).

All chemicals were reagent grade or better. So that contamination by divalent metal ions could be avoided, sodium chloride for preparation of buffers was ultrapure grade (Alfa-Ventron, Danvers, MA). Except as noted, chloride salts were used to prepare 1 mM stocks of the following metal ions; Mn^{2+} (Fisher ACS Reagent), Cd^{2+} (Mallinckrodt Reagent), Ca^{2+} (Fisher Reagent; heavy metals <0.0004%), Mg^{2+} (Fisher ACS Reagent; Mn^{2+} <1 ppm), Ti^{2+} (as sulfate, MCB), Pr^{3+} (Alfa-Ventron), and Eu^{3+} (Alfa-Ventron). Water for buffers was distilled from basic potassium permanganate solution and then redistilled from a Pyrex container, saturated with argon, and stored in borosilicate glass containers under argon at 4 °C until use. Sodium cacodylate [$Na^+[(CH_3)_2As\ O(OH)]^-$] was purchased from Polysciences, Inc. (Warrington, PA; lots 01739, 03456) and used as an anhydrous powder. As noted previously (Lentz et al., 1980), the choice of cacodylate as a buffer was dictated by the susceptibility of the common amine base buffers to periodate oxidation. We and others (Szoka et al., 1980) have used a phosphate buffer system with our assay procedure, but with somewhat more rapid loss of bilayer permeability barrier during oxidation than achieved with a cacodylate buffer. Unfortunately, the use of sodium cacodylate in a buffer system is not without problems. Several lots of sodium cacodylate produced buffer that gave high blanks in our assay systems. Two lots produced buffer in which it was difficult to maintain a permeability barrier across the SUV membranes. These problems could be surmounted by recrystallizing the sodium cacodylate from methanol/benzene. The impure salt was dissolved hot in a minimal amount of methanol. The hot solution was filtered, leaving a yellow residue on the filter paper. Benzene was added at room temperature until a cloud point was achieved. Crystals were allowed to form slowly overnight at 4 °C. The supernatant was decanted off, and the crystals were dried under vacuum and then stored in a dry argon environment until used.

Preparation of Small, Unilamellar Vesicles. Mixtures of DMPC with $DC_{15}PG$ or of egg PG with egg PC were prepared in chloroform/methanol and dried under a stream of argon as a thin film onto the wall of an annealed glass ampule (Kimble, 10 mL). The sample was further dried under high vacuum for 6–12 h. Buffer (100 mM NaCl and 50 mM sodium cacodylate, pH 7.2) was added to the ampule, and the phospholipids were dispersed to a final concentration of 2–10 mM in total phospholipid (depending on the PG content of the sample). Phospholipid mixtures were suspended and sonicated at temperatures (10 °C for egg PG/egg PC mixtures and 43 °C for $DC_{15}PG$/DMPC mixtures) well above the highest phase transition temperature of either component. Phospholipid suspensions were sonicated directly in the glass ampule by using a Heat Systems W350 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) equipped with a Heat Systems Cup Horn (Barrow & Lentz, 1980). In this procedure, the sonic energy is transmitted into the sample through the walls of the glass ampule, and the sample never contacts a metal surface. For comparison, several samples were sonicated by the classical procedure (Huang, 1969) in which a $^3/_8$-in. Heat Systems titanium tip is directly immersed in the sample. Sonicated preparations were fractionated (Barenholz et al., 1977) by centrifugation in a Beckman Ti75 rotor at 40–48 °C for $DC_{15}PG$/DMPC and at 5–12 °C for egg PG/egg PC at a speed of 49 000 rpm for 1 h (Lentz et al., 1980). Region III vesicles (Barenholz et al., 1977) were stored above their phase transition (at 10 °C for egg PG/egg PC and at 43 °C for $DC_{15}PG$/DMPC) until use (usually within 1 h).

Assay for Phosphatidylglycerol Transbilayer Distribution in the Presence of Different Metal Ions. SUV preparations were diluted before assay to a phosphatidylglycerol concentration of 0.3 mM. For experiments other than kinetic experiments, microliter aliquots of metal ion stocks were added to 3–5 mL of SUV preparation and allowed to incubate at 10–12 (egg PG/egg PC) or 42 °C ($DC_{15}PG$/DMPC) for 2.5 h. A small volume of Na_2EDTA sufficient to fully complex (<10 ppm free ion) the added di- or trivalent metal ions was added to each sample before it was assayed at 42 °C. Each sample was distributed into 10–15 culture tubes (0.3 mL/tube) and assayed for *exposed* and *total* phosphatidylglycerol as described previously (Lentz et al., 1980). The contents of different tubes were oxidized with potassium periodate for different lengths of time so as to follow the time course of the oxidation reaction. After quenching each oxidation reaction with sodium bisulfite, the formaldehyde released by the oxidation of glycerol was determined spectrophotometrically by reaction with chromotropic acid. As previously reported (Lentz et al., 1980), there was a time period during which both the exposed and total phosphatidylglycerols were found to remain at a constant level. Following this "plateau period", the exposed phosphatidylglycerol approached the "total" value in concert with the breakdown in vesicle permeability barrier (Lentz et al., 1980). The reported ratio of exposed to total phosphatidylglycerol (PG_{exp}/PG_{tot}) was taken from this plateau period, which had to include at least three and preferably four

or five time points in order to be considered a valid plateau. For each ion study, an identical sample was incubated and assayed without the addition of metal ion as a control.

As an aside, we note another problem which initially impeded this study. Under the conditions required to react formaldehyde with chromotropic acid (18 N H_2SO_4 at 100 °C), sodium dodecyl sulfate (required to disrupt SUV membranes in the total assay) was transformed into an oily material which was nearly impossible to remove from the walls of the culture tube by normal washing. Only boiling of the culture tubes in dilute sodium hypochlorite (1 part Clorox to 100 parts water) followed by copious rinsing with distilled water resulted in reproducible assay values.

Thin-Layer Chromatography of Oxidized Phosphatidylglycerol. Lipids were extracted from oxidized 50 mol % $DC_{15}PG$/DMPC vesicles (6-, 12-, 15-, 18-, 23-, and 40-min oxidation times) by the procedure of Bligh & Dyer (1959) with the addition of HCl (to 0.005 M) and spotted on Analtech (Newark, DE) uniplates precoated (0.25 mm) with silica gel GHL containing 0.01 M dipotassium oxalate as binder. The thin-layer plates were developed with both chloroform/methanol/water/acetic acid (65:25:2.8:2 v/v) and chloroform/methanol/water (65:25:4). Thin-layer plates were stained with I_2 vapors, and the spots were scraped into 18-mm test tubes and analyzed for phosphate by the method of Chen et al. (1956).

Nuclear Magnetic Resonance (NMR) Experiments. SUV for ^1H NMR measurements were prepared by sonicating 1 mL of a 20–40 mM lipid suspension in a 10-mL polycarbonate centrifuge tube in short bursts (Barrow & Lentz, 1981). The preparation was fractionated by centrifugation for 90 min in the type A-100 rotor of a Beckman Airfuge at a speed of 84 000 rpm. The sonication buffer was 100 mM NaCl and 1 mM sodium phosphate, pH 7.2, instead of the proton-containing cacodylate buffer. Samples were dialyzed against an equivalent buffer prepared with D_2O (KOR Inc., Cambridge, MA; 99.8 mol % isotopic purity; conductivity <0.1 $m\Omega^{-1}/m$, equivalent to doubly distilled water). Samples were transferred into 5-mm NMR tubes under an argon atmosphere and sealed into the tube with a Teflon chuck (Wilmad, Buena, NJ). Low-resolution spectra were taken on a Varian EM3930 spectrometer operating at 90 MHz, while high-resolution spectra were obtained on a Bruker Cryospec 250 WM FT NMR instrument operating at 250 MHz. The low-resolution spectra were obtained in a single sweep in continuous wave mode, while the high-resolution spectra were collected in 40–100 sweeps in fourier transform mode with quadrupole decoupling and a lock on ^2H.

Electron Microscopy. Negative-staining electron microscopy was employed to gauge morphological changes associated with different treatments of PG-containing vesicles (Valentine et al., 1968). A carbon-coated mica sheet was dipped into the vesicle suspension, and the carbon layer was floated onto 2% ammonium molybdate. The carbon layer was lifted from the ammonium molybdate staining solution on a 400-mesh copper grid (Polysciences Inc., Warrington, PA), which was removed to dry on filter paper. Preparations were examined within 2–3 h on a JEOL 100 B electron microscope operated at 80 kV.

Results

Effect of Mn^{2+} on the Transbilayer Distribution of PG As Determined by Chemical Modification. Previously, we have shown that 80% of total PG in 50 mol % $DC_{15}PG$/DMPC vesicles was oxidized by periodate through the end of the plateau period for the oxidation reaction (Lentz et al., 1980). In Figure 1, we show that the fraction of PG oxidized dropped

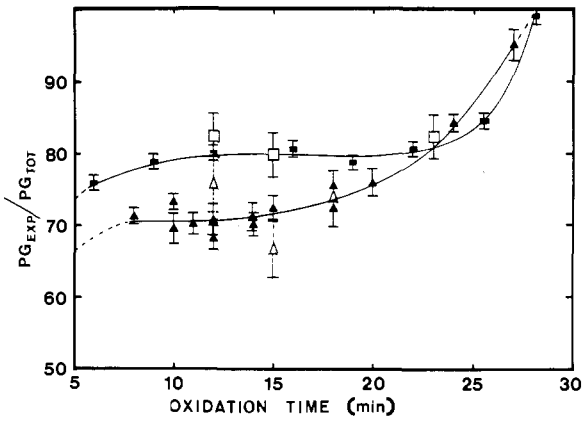

FIGURE 1: Time dependence of the ratio of exposed (oxidized in intact small, unilamellar vesicles) to total (oxidized in $NaDodSO_4$-solubilized vesicles) phosphatidylglycerol obtained by the chromogenic assay in $DC_{15}PG$/DMPC vesicles free of Mn^{2+} (■) and incubated at 100 phospholipids/Mn^{2+} for 2.5 h before assay (▲). The ratio of the amount of oxidized phospholipid to total initial phosphatidylglycerol as determined by thin-layer chromatographic analysis is also given for a Mn^{2+}-free (□) and Mn^{2+}-containing (△) sample. Error bars (standard deviations) were calculated by using standard error analysis methods from the errors inherent in the chromogenic and phosphate (for TLC analysis) assays.

to 70% when vesicles were incubated in the presence of less than 1 mol % Mn^{2+} before oxidation. A similar drop in the fraction oxidized was observed in egg PG/egg PC mixtures and in both lipid systems at different molar contents of PG. We wished to check whether this observation might be due to complex surface reactions resulting in different oxidation products in the presence or absence of Mn^{2+}. Therefore, we analyzed the assay mix by thin-layer chromatography (see Materials and Methods) to determine if the product of the oxidation reaction was altered by the presence of Mn^{2+}. In both an acidic and a neutral solvent system, only one additional spot was detected, beyond those corresponding to pure PG and PC, when samples were oxidized either in the presence or in the absence of Mn^{2+}. This spot had an R_f of 0.68 in chloroform/methanol/water/acetic acid (65:25:2.8:2) and 0.44 in chloroform/methanol/water (65:25:4). Other R_f values were the following: PC, 0.14 and 0.16, respectively; PG, 0.37 and 0.28, respectively. These results demonstrate that the same single compound was formed by periodate oxidation in the presence and absence of Mn^{2+}. The amount of material associated with each spot was determined by phosphate assay (Chen et al., 1956) and used to calculate the PG_{exp}/PG_{tot} ratios which are plotted in Figure 1. Within the limits of precision, the thin-layer analysis agreed well with the chromogenic development of the oxidation reaction (Figure 1). This indicates that the detection of formaldehyde by chromotropic acid development accurately reflects this chemical modification process. The presence of free Mn^{2+} at the time of the assay was not necessary for the reduction in oxidizable PG, since EDTA added just prior to the oxidation had no effect on results obtained in samples containing or not containing Mn^{2+}. However, the presence of free Mn^{2+} during the incubation period was essential, since inclusion of EDTA before addition of Mn^{2+} led to results indistinguishable from those for a Mn^{2+}-free sample.

A question remains as to whether either Mn^{2+} or the oxidation process might disrupt the SUV structure in such a way as to explain the results shown in Figure 1. For example, fusion to form large vesicles would lead to a reduction of exposed phosphatidylglycerol simply due to a decrease in exposed total lipid with increased vesicle size. Several lines of

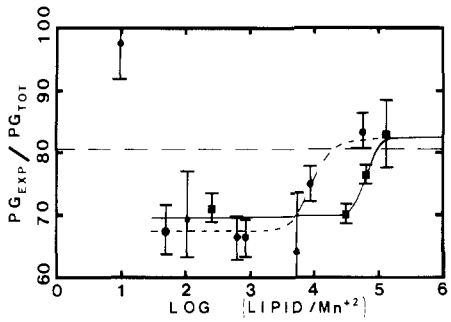

FIGURE 2: Dose response of the effect of Mn^{2+} on the ratio of exposed to total phosphatidylglycerol (PG_{exp}/PG_{tot}) in 50 mol % $DC_{15}PG$/DMPC SUV (0.2 mM total lipid) incubated at the indicated lipid to Mn^{2+} ratios for 2 (●) and 2.5 (■) h before addition of Na_2EDTA (to 0.03 mM) and assay as illustrated in Figure 1. The ratio of exposed to total phosphatidylglycerol without the addition of Mn^{2+} is given by the dashed horizontal line (--).

evidence argue against this possibility. First, periodate oxidation or treatment with low levels of Mn^{2+} did not alter the permeability properties of the vesicles and, therefore, presumably did not disrupt their structure. This was demonstrated by several observations. For instance, Mn^{2+} did not induce its own permeability through the vesicle bilayer, as ^1H NMR spectra of 50 mol % egg PG/egg PC did not change over a period of 35 min, even at a ratio of only 119 lipids/Mn^{2+} (see below). This is in agreement with the ^{31}P NMR results of Nordlund et al. (1981). In addition, periodate oxidation did not cause a change in vesicle permeability to carboxyfluorescein, at least through the end of the level period (Lentz et al., 1980). Second, exposure to Mn^{2+} produced no change in vesicle size as judged by observation of a constant-intensity, sharp ^1H NMR choline peak over a period of 35 min. It is commonly observed that fusion to produce large vesicles produces line broadening and a resultant intensity loss in the sharp choline peak (Prestegard & Fellmuth, 1974; Schmidt et al., 1981). In addition, PG/PC SUV exposed to small amounts of Mn^{2+} showed Rayleigh scattering behavior identical with their behavior before addition of Mn^{2+}. Such light scattering measurements are capable of detecting as little as 1 mol % large, multilamellar vesicles in a SUV preparation (Barrow & Lentz, 1980). Third, periodate oxidation through the level period or exposure to low concentrations of Mn^{2+} (ca. 100 lipids/Mn^{2+}) produced no alteration in vesicle morphology relative to untreated vesicles, as judged by electron micrographs of negatively stained preparations.

While most of the results described above were obtained at a lipid/Mn^{2+} ratio of about 100/1, we have found the reduction in exposed PG to occur over a wide range of Mn^{2+} concentrations. Figure 2 records observed PG_{exp}/PG_{tot} as a function of the ratio of the number of lipid molecules to Mn^{2+} ions in the incubation mixture before the oxidation assay was performed. At very high levels of ion, all PG becomes exposed, presumably due to loss of the membrane permeability barrier. Over a wide range of intermediate ion levels, we observed the same decrease in exposed PG reported above. Quite surprisingly, this effect persisted down to extremely low levels of Mn^{2+}, even at a low concentration of lipid (see Figure 2). After about 2-h incubation, as little as one Mn^{2+} per 10000 lipids had induced a change in the fraction of exposed PG. A longer incubation time somewhat reduced the amount of Mn^{2+} required to produce reorientation (Figure 2).

The observation of a shift in the dose–response curve for a change in incubation time (see above; Figure 2), as well as our NMR results (see below), suggested that Mn^{2+}-induced lipid redistribution might be slow enough to follow kinetically

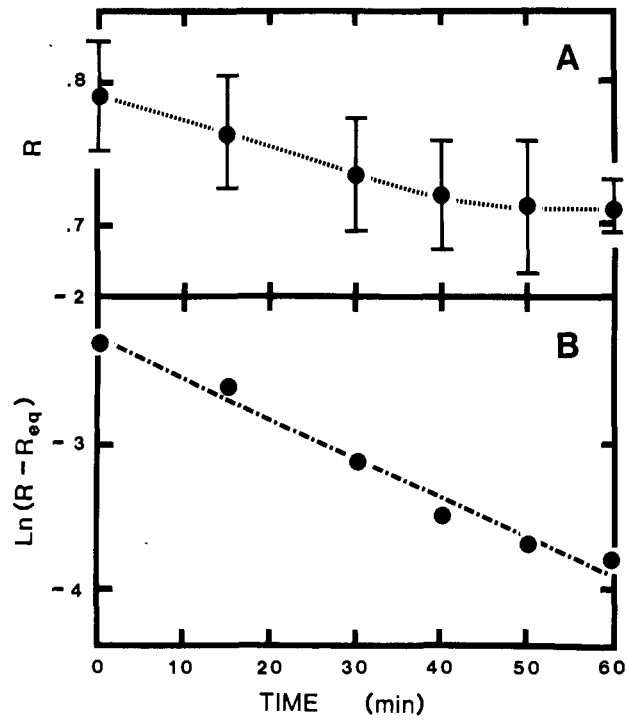

FIGURE 3: (A) Time dependence of the decay of phosphatidylglycerol asymmetry in 50 mol % $DC_{15}PG$/DMPC small vesicles (0.22 mM total lipid) in the presence of 10000 lipids per externally added Mn^{2+}. The effect of Mn^{2+} was quenched at different times by addition of 0.3 mM Na_2EDTA, resulting in exposed to total PG ratios (R) intermediate between 0.79 (R_0) and 0.69 (R_{eq}). (B) The data from (A) are logarithmically transformed and plotted according to the first order expression shown in eq 1, derived for the process shown in eq 2, where $K = k_1 + k_2$. The value of K obtained in this fashion was 0.027 ± 0.016 min^{-1}.

$$R - R_{eq} = (R_0 - R_{eq})e^{-Kt} \quad (1)$$

$$PG_{out} + PC_{in} \underset{k_2}{\overset{k_1}{\rightleftarrows}} PG_{in} + PC_{out} \quad (2)$$

at low Mn^{2+} concentrations. Figure 3A shows data for the time dependence of the exposed to total phosphatidylglycerol ratio in 50% $DC_{15}PG$/DMPC vesicles to which was added 1 g atomic weight of Mn^{2+} per 10000 g molecular weight of total phospholipid at time zero. Following incubation at 40 °C for the times indicated on the abscissa, Na_2EDTA was added to complex the Mn^{2+}, and the vesicle preparation was assayed by our oxidation assay procedure. The exponential time constant so obtained from the slope of the logarithmically transformed data (Figure 3B) was 24–94 min. These data unambiguously demonstrate that the loss of PG exposure on the vesicle surface is directly related to exposure to Mn^{2+}.

NMR Determination of Transbilayer Lipid Distribution. In order to test by another method the effect of Mn^{2+} on transbilayer lipid distribution, we turned to magnetic resonance spectroscopy, a technique which has been widely used to estimate lipid asymmetry in model membranes (Michaelson et al., 1973; Berden et al., 1975; Barsukov et al., 1980; Nordlund et al., 1981). In this method, transition-state or lanthanide-series ions are used either to broaden or to shift the signal from nuclei associated with the outer leaflet of a vesicle bilayer. The most common and effective broadening agent is Mn^{2+}, which is unfortunately the ion whose effect on lipid distribution we wished to test. The common shift reagents are trivalent lanthanide-series ions (e.g., Pr^{3+} or Gd^{3+}) which we have found to expose all vesicle PG to oxidation at the lipid/ion ratio needed in NMR shift experiments (ca. 10 lipids/ion; Shaw et al., 1977). With these limitations in mind, we decided to

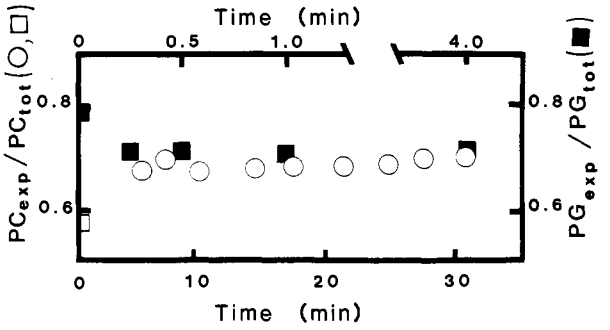

FIGURE 4: Time dependence of the fraction of outer-monolayer phosphatidylcholine (PC_{out}/PC_{tot}; open symbols) and of the fraction of exposed phosphatidylglycerol (PG_{exp}/PG_{tot}; shaded) in 50 mol % egg PG/egg PC vesicles. The NMR results (○) were obtained on a Varian EM 390 NMR spectrometer with acetic acid contained in a capillary as an internal intensity standard. The intensity associated with the choline $N^+(CH_3)_3$ peak was measured relative to the standard before adding Mn^{2+} and then at a series of times following Mn^{2+} addition, allowing the calculation of PC_{out}/PC_{tot}. Standard deviations based on averaging five independent measurements were 3–4%. The NMR data are plotted vs. the lower time axis. For comparison, a value of PC_{out}/PC_{tot} (□) (calculated standard deviation 5%) was derived from chemical modification data on the same sample before Mn^{2+} addition, assuming the fraction of total phospholipid in the outer membrane leaflet was 0.69 (see text). Data obtained by the chemical modification procedure with a similar 50 mol % PG/PC sample are plotted vs. the upper time axis as PG_{exp}/PG_{tot} (■) (standard deviations 2–3%). A measurement performed before the addition of Mn^{2+} is plotted at zero time. Lipid and Mn^{2+} concentrations were respectively 21 and 0.20 mM, and 40 and 0.48 mM for the NMR and chemical modification experiments, respectively.

follow the choline ^1H signal of egg PC in 50 mol % egg PG/egg PC vesicles as a function of time following addition of Mn^{2+}. The results of this experiment are shown in Figure 4. No change in the ratio of outer leaflet to total phosphatidylcholine was observed during the time interval from 4 to 31 min following the addition of Mn^{2+} [Figure 4 (○)]. The average value of PC_{out}/PC_{tot} (0.69 ± 0.01) agrees with the value of 0.69 ± 0.06 derived from the ^{31}P NMR data of Nordlund et al. (1981) and indicates a symmetric distribution of egg PC across the vesicle bilayer. This result differs with results obtained on the same SUV sample by chemical modification of exposed PG *in the absence of* Mn^{2+}. The result of this chemical modification experiment is plotted in Figure 4 at zero time [i.e., indicating no Mn^{2+} added; Figure 4 (□)]. Expression of assayed PG_{exp}/PG_{tot} values as PC_{out}/PC_{tot} (as obtained from NMR) required the assumption that the ratio of total lipid in the outer monolayer to that in the inner monolayer was 0.69. This value is consistent with the known size of egg PC vesicles (Barenholz et al., 1977). There are two plausible explanations for the difference between the assay and NMR results. It could be that the chemical modification procedure perturbed bilayer asymmetry, thereby giving a result different from that obtained by NMR techniques. Alternatively, it may be that Mn^{2+} induced a change in lipid transbilayer distribution more rapidly than could be detected with the first NMR measurement taken 3.9 min after addition of Mn^{2+} (Figure 4). Since this was the earliest time point obtainable by the NMR measurement, we decided to investigate shorter time periods with our chemical modification procedure, using EDTA to complex free Mn^{2+} at various times after its addition to the vesicle preparation. The results are plotted in Figure 4 (■) as the fraction of exposed phosphatidylglycerol (PG_{exp}/PG_{tot}) vs. time on the upper abscissa. Also plotted in Figure 4 is the fraction of exposed phosphatidylglycerol observed for the same vesicle sample before the additions of Mn^{2+} (point at time zero). Two major conclusions follow from these

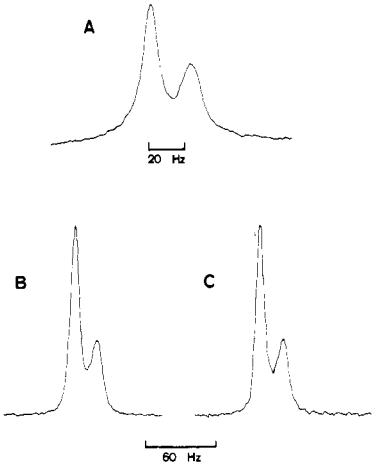

FIGURE 5: (A) High-resolution nuclear magnetic resonance spectrum of the choline methyl protons in a 50 mol % $DC_{15}PC$/DMPC SUV preparation (40 mM) obtained at 40 °C on a Bruker 250-MHz instrument. The chemical shifts of the choline peaks measured relative to the carboxyl proton of acetic acid were −7.28 and −7.35. (B) Similar choline spectrum of a similar sample (14.1 mM) incubated with Mn^{2+} before dialysis against D_2O–phosphate buffer (see text). (C) Spectrum of a similar sample (14.3 mM) prepared from DMPC not washed with EDTA solution. This sample was not incubated with Mn^{2+}.

data. First, the results obtained by the assay procedure after the addition of Mn^{2+} are in essential agreement with the lack of asymmetry observed by the NMR technique in 50 mol % egg PG/egg PC vesicles. This is particularly evident if the PG_{exp}/PG_{tot} data obtained by chemical modification are transformed to the form PC_{out}/PC_{tot} (assuming [outside total lipid]/[inside total lipid] = 0.69, as above) to give an average value of 0.67 ± 0.03, which compares favorably with the value from the NMR data of 0.69 ± 0.025. We further conclude that the loss of bilayer asymmetry induced by Mn^{2+} must take place in less than 15 s, at least at the concentrations of lipid and Mn^{2+} used for the experiments summarized in Figure 4.

Since it proved impossible to perform an NMR experiment rapidly enough to detect the effect of Mn^{2+} on transbilayer lipid distribution, we attempted a high-resolution NMR experiment with the hope of resolving the phosphatidylcholine transbilayer distribution in the absence of Mn^{2+} or shift reagents. Figure 5A shows that the high-resolution spectrum does resolve choline ^1H signals from the two membrane leaflets but not sufficiently to allow direct measurement of individual peak areas. In order to circumvent the incomplete resolution of inner and outer leaflet signals, we took advantage of the theoretical Lorentzian shapes of the NMR spectra. The essential characteristics of a Lorentzian curve (peak height, I_{max}, and width at half-maximum, $\Delta\omega_{1/2}$) were determined from the spectrum in Figure 5A and used to calculate peak areas according to the formula, area = $\pi\Delta\omega_{1/2}I_{max}$. Peak areas obtained in this way yielded a ratio of outer to inner leaflet phosphatidylcholine (PC_{out}/PC_{in}) of 1.57 ± 0.03 (Table I). This value is considerably smaller than the value of 2.1–2.2 obtained by Nordlund et al. (1981) for 50 mol % egg PG/egg PC vesicles and clearly indicates an asymmetric distribution of phosphatidylcholine. In order to compare directly with assayed values of PG_{exp}/PG_{tot}, we again assumed a value of 0.69 for the ratio of outer to inner leaflet total lipid. PG_{out}/PG_{tot} calculated from the NMR results was found to agree well with PG_{exp}/PG_{tot} determined for the same sample by the chemical modification procedure in the absence of Mn^{2+} (Table I). So that the effect of exposure to Mn^{2+} could be tested, a similar sample was incubated with Mn^{2+} (lipid/Mn^{2+} ratio = 5000/1) for 15 min prior to dialysis against D_2O-based

Table I: Summary of High-Resolution NMR Results

conditions for NMR expt[a]	PC_{out}/PC_{in}[b]	PG_{out}/PG_{tot} from NMR[b,c]	PG_{exp}/PG_{tot} from assay[d]
no Mn^{2+}, Figure 5A	1.57 ± 0.06	0.77 ± 0.04	0.79 ± 0.02
1 Mn^{2+}/5000 lipid Figure 5B	2.17 ± 0.08	0.70 ± 0.05	0.69 ± 0.02
no Mn^{2+}, unwashed DMPC, Figure 5C	2.35 ± 0.09	0.68 ± 0.05	0.69 ± 0.02

[a] All experiments were performed with 50 mol % $DC_{15}PG$/DMPC SUV. Further details are given in the legend to Figure 5. [b] Error ranges were derived from the estimated errors involved in measuring peak widths and heights. [c] In order to derive PG_{out}/PG_{tot} from PC_{out}/PC_{in} measured directly from the NMR spectra, we assumed a value for the ratio of outer to inner leaflet total lipid equal to that for egg PC (Barenholz et al., 1977). This is reasonable since $DC_{15}PG$/DMPC vesicles were indistinguishable in size from pure egg PC vesicles (Lentz et al., 1980). [d] Error ranges were obtained as the standard deviation of duplicate measurements on each of four assays performed during the plateau period of PG oxidation (see Figure 1).

buffer. The ^1H NMR spectrum of this sample (Figure 5B) yielded a PC_{out}/PC_{in} ratio from which we derived PG_{out}/PG_{tot} in excellent agreement with assay results for the same sample (Table I). Both results were indicative of a symmetric distribution, indicating that incubation with Mn^{2+} had induced lipid transbilayer redistribution. It should be noted that the conditions required for NMR experiments (high concentration of lipid, extensive dialysis against D_2O) made it difficult to control ionic environment. Experiments with EDTA in the buffer were attempted, but a resonance of the chelating agent interfered with the choline methyl resonance of PC. Because of the high lipid concentrations, only minute quantities of Mn^{2+} should be required to cause transbilayer lipid rearrangement (see Discussion of redistribution kinetics). For this reason, both $DC_{15}PG$ and DMPC lipid stocks had to be washed with EDTA solution (see Materials and Methods), and dialysis bags had to be soaked and briefly sonicated in warm EDTA solution before use. Figure 5C shows the choline region of a spectrum obtained on a sample for which the EDTA wash of the DMPC stock was not carried out. The PC_{out}/PC_{in} ratio from this spectrum yielded PG_{out}/PG_{in} characteristic of a symmetric distribution and in agreement with the value obtained by our chemical modification procedure (Table I). This sensitivity to divalent metal ion contaminants makes it difficult to detect PG asymmetry by NMR even without using Mn^{2+} and could explain the failure of Nordlund et al. (1981) to detect asymmetry using potassium ferricyanide as a shift reagent. Despite such difficulties, the phosphatidylglycerol transbilayer distribution calculated from NMR spectra agreed in every case with that obtained directly by chemical assay of the same sample. These results provide compelling evidence for the validity of our chemical assay and support our conclusion that low doses of Mn^{2+} induce transbilayer redistribution of lipids in mixed phosphatidylglycerol/phosphatidylcholine SUV.

Effects of Other Ions on Transbilayer Distribution of Phosphatidylglycerol. Several divalent and trivalent metal ions besides Mn^{2+} were tested for their effect on the distribution of phosphatidylglycerol across the bilayer membranes of SUV. In addition to Mn^{2+}, only Cd^{2+} was found to induce the transbilayer redistribution of lipids (see Table II). These two ions were effective in both $DC_{15}PG$/DMPC and egg PG/egg PC mixtures.

Transbilayer Redistribution of Phosphatidylglycerol as a Function of Vesicle Composition. We have previously reported that the transbilayer distribution of PG in $DC_{15}PG$/DMPC

Table II: Effect of Multivalent Metal Ions on Phosphatidylglycerol Exposure in Small, Unilamellar Vesicles

lipid mixture (mol % PG)	ion	total lipid/ion (mol/mol)	PG_{exp}/PG_{tot} without ion[a]	PG_{exp}/PG_{tot} with ion[a,b]
50% $DC_{15}PG$/DMPC	Mn^{2+}	100/1	0.80 ± 0.02	0.69 ± 0.04
15% egg PG/egg PC	Mn^{2+}	100/1	0.91 ± 0.13	0.81 ± 0.02
50% $DC_{15}PG$/DMPC	Cd^{2+}	100/1	0.80 ± 0.03	0.73 ± 0.02
15% egg PG/egg PC	Cd^{2+}	100/1	0.91 ± 0.03	0.81 ± 0.02
50% $DC_{15}PG$/DMPC	Ca^{2+}	100/1; 1000/1	0.81 ± 0.03	0.80 ± 0.02
50% $DC_{15}PG$/DMPC	Mg^{2+}	100/1	0.81 ± 0.03	0.84 ± 0.03
50% $DC_{15}PG$/DMPC	Ti^{2+}	100/1	0.81 ± 0.03	0.77 ± 0.04
50% $DC_{15}PG$/DMPC	Co^{2+}	150/1	0.79 ± 0.02	0.77 ± 0.02
50% $DC_{15}PG$/DMPC	Ni^{2+}	150/1	0.79 ± 0.02	0.77 ± 0.02
50% $DC_{15}PC$/DMPC	Pr^{3+}	237/1; 1000/1	0.80 ± 0.02	0.79 ± 0.02
15% egg PG/egg PC	Pr^{3+}	100/1	0.90 ± 0.02	0.91 ± 0.02
50% $DC_{15}PG$/DMPC	Eu^{3+}	400/1	0.78 ± 0.04	0.82 ± 0.02

[a] Error ranges were obtained as the standard deviation of duplicate measurements on each of three to eight (usually four or five) assays performed during the plateau period of PG oxidation (see Figure 1). In most cases, the standard deviations in both the exposed PG and total PG measurements were 1-1.5%, consistent with the listed error ranges in the ratio of exposed to total PG. [b] Lipid vesicles were incubated with ions at 40-42 °C for 2.5 h before addition of EDTA prior to assay by the chemical modification procedure described under Materials and Methods.

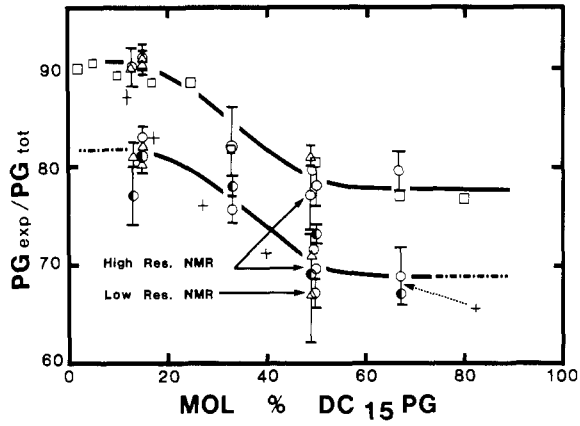

FIGURE 6: Dependence of the ratio of exposed to total phosphatidylglycerol (PG_{exp}/PG_{tot}) on SUV composition in the absence (open symbols, upper curve) and presence of Mn^{2+} (open symbols, lower curve) or Cd^{2+} (half-shaded symbols, lower curve). Vesicles composed of $DC_{15}PG$/DMPC (circles) or egg PG/egg PC (triangles) mixtures were assayed for phosphatidylglycerol as described in the text. Data for $DC_{15}PG$/DMPC mixtures previously studied (Lentz et al., 1980) are reproduced for comparison (□). Also shown for comparison are data derived from Figure 5 of Massari et al. (1978) for egg PG/dipalmitoylphosphatidylcholine mixtures in SUV preparations (+). Values of outer leaflet to total phosphatidylglycerol derived from nuclear magnetic resonance data are presented for comparison and labeled "High Res. NMR" (250 MHz; Figure 5) and "Low Res. NMR" (90 MHz). Error bars generally represent one standard deviation as calculated from errors in the measured values. Uncertainties in the composition were typically less than 2 mol %.

SUV varied with vesicle composition (Lentz et al., 1980). We have now found that small quantities of Mn^{2+} and Cd^{2+} induce redistribution of phosphatidylglycerol in vesicles of all compositions studied (Figure 6). However, only at or above 40 mol % phosphatidylglycerol did the ultimate ratio of exposed to total PG approach that characteristic of a random transbilayer distribution of lipids (ca. 0.69). At lower PG contents, the charged phosphatidylglycerol species distributed to the outer bilayer leaflet of SUV even in the presence of Mn^{2+}, although less so than in its absence. In the absence of Mn^{2+}

Table III: Effect of Sonication with an Immersed Titanium Tip on Phosphatidylglycerol Exposure in Small, Unilamellar Vesicles

lipid mixture (mol % $DC_{15}PG$/DMPC)	conditions of sonication	PG_{exp}/PG_{tot}
50%	15 1-min pulses with a titanium tip at 45 °C	0.89 ± 0.03
50%	27 1-min pulses with a titanium tip at 40 °C in buffer containing 0.3 mM Na_2EDTA	0.67 ± 0.04
50%	1.5-h continuous sonication in a 5-mL glass ampule placed in the Heat Systems Cup Horn at 45 °C	0.80 ± 0.02
15%	13 0.5-min pulses with a titanium tip at 46 °C in buffer containing 0.05 mM Na_2EDTA	0.76 ± 0.01
15%	1-h continuous sonication in a 5-mL glass ampule placed in the Heat Systems Cup Horn at 42 °C	0.91 ± 0.01

or Cd^{2+}, the present results agree perfectly with the results we had previously published (Lentz et al., 1980; Figure 6). In addition, we have extended our earlier studies demonstrating that egg PG/egg PC vesicles show the same PG transbilayer distribution as observed for the saturated, synthetic $DC_{15}PG$/DMPC mixtures (Figure 6). These findings are all summarized in Figure 6. For comparison, we have also plotted in Figure 6 the ratios of outer leaflet to inner leaflet PG derived from our NMR experiments performed in the presence and absence of Mn^{2+}.

Effect of Titanium-Tip Sonication on Transbilayer Phosphatidylglycerol Distribution. Given the significant effect of divalent metal ions on phosphatidylglycerol transbilayer distribution, we wondered whether exposure to a titanium sonication tip during the sonication process might produce vesicles with a different lipid distribution than our cup-sonicated vesicles (see Materials and Methods; Lentz et al., 1980; Barrow & Lentz, 1980). Table III records the results of duplicate tests to determine the effects of exposure to a titanium tip during sonication. Although Ti^{2+} had no effect on PG exposure (Table I), sonication with a titanium tip clearly resulted in exposure of more PG than did sonication with the Cup Horn. By contrast, if some Na_2EDTA was added to the sonication buffer, the measured exposed-to-total PG ratio was characteristic of a random distribution of lipids, at least at 50 mol % PG. This agrees with the lipid distributions reported by Nordlund et al. (1981), who also included EDTA in their sonication buffer. At 15 mol % PG, titanium-tip sonication in the presence of EDTA produced vesicles having a slight disproportionation of PG to the vesicle outer leaflet (Table III), similar to our observations on cup-sonicated vesicles treated with Mn^{2+} (Figure 6). While these results do not suggest a mechanism to explain the effect of titanium-tip sonication, they do sound a warning that the conditions present during the sonication process dramatically affect the transbilayer distribution achieved in an SUV preparation.

Discussion

The distribution of charged lipids within the bilayer of a biological membrane should be an important factor in determining membrane functional properties. Membrane fusion, platelet–membrane clot promotion, and the binding of extrinsic membrane proteins all involve acidic phospholipids and are processes for which the transbilayer distribution of these lipids should have considerable significance. Unfortunately, the principles guiding the transbilayer distribution of charged lipids even in model membranes are in question due to differences between the results obtained by NMR (Berden et al., 1975; Barsukov et al., 1980; Koter et al., 1978; Nordlund et al., 1981) and chemical modification or dye-binding techniques (Massari et al., 1978; Lentz et al., 1980). A principal motivation in undertaking the current study was to seek an explanation for some of these discrepancies. We have uncovered at least two possible sources of differences. First, the divalent metal ions often used to broaden spin resonance lines in NMR studies also perturbed the transbilayer distribution in PG/PC SUV. Such broadening reagents were used in the studies of Koter et al. (1978) and Nordlund et al. (1981), who concluded that phosphatidic acid and phosphatidylglycerol, respectively, were randomly distributed between membrane leaflets in phosphatidylcholine-containing SUV. In the presence of Mn^{2+}, our chemical modification results for PG distribution agree with the NMR results of Nordlund et al. at least for vesicles containing greater than ca. 40 mol % PG. The presence of Mn^{2+}, however, does not account for our continued difference with the results of Nordlund et al. in the range 10–40 mol % PG. This difference may be due to the second source of discrepancy we have found, namely, that the transbilayer distribution was sensitive to the sonication procedure used to produce small vesicle dispersions. Sonication with an immersed titanium tip in the presence of EDTA produced vesicles having a nearly symmetric distribution of PG at both low PG content and high PG content (Table III). In our experience, it would be very difficult to distinguish between a completely symmetric and slightly asymmetric distribution of phosphatidylcholine by NMR methods at low PG contents. Thus, the combination of these two surprising effects can explain completely the differences between results obtained by our chemical procedure and the careful NMR studies of Nordlund et al. (1981).

Taken together, the experiments presented here strongly implicate Mn^{2+} and Cd^{2+} as *catalysts* in the redistribution of phosphatidylglycerol (and, by implication, phosphatidylcholine) between the outer and inner leaflets of SUV membranes. Several observations suggest this. First, less than one Mn^{2+} per phospholipid vesicle is sufficient to bring about redistribution if sufficient time is allotted (see Figure 2). Second, the Mn^{2+} does not need to enter the closed compartment of the vesicle in order to have its effect, as evidenced by the constancy of our NMR spectra with time [Figure 4; also see Nordlund et al. (1981)]. Third, Mn^{2+} did not induce any gross structural alterations (other than transbilayer redistribution) in the vesicles, at least as judged by light scattering (Barrow & Lentz, 1980), NMR spectroscopy, negative-staining electron microscopy, and gel chromatography (Nordlund et al., 1981). Finally, at high concentrations of lipid and ion, the ion-induced redistribution is extremely rapid (time constant <7 s; see Figure 4). All of this suggests that the Mn^{2+} acts not as a driving force for the redistribution reaction but rather only to lower the activation barrier slowing spontaneous approach to equilibrium. It follows that the lipid transbilayer distribution observed in Cup Horn sonified PG/PC SUV is not an equilibrium distribution. This agrees with our observation that the exposed-to-total PG ratios were sensitive to sonication procedure (see Table II). These results imply that information about equilibrium lipid packing arrangements may not be obtainable from SUV asymmetry investigations. Indeed, even the presence of Mn^{2+} does not guarantee attainment of an equilibrium lipid distribution but might result only in the formation of a more stable and different intermediate. Nonetheless, two facts argue in favor of this being an equilibrium distribution. First, roughly the same distribution was

obtained by addition of Mn^{2+} to Cup-Horn sonified samples as by titanium-tip sonication in the presence of EDTA (Table III and Figure 6). Second, a similar distribution [see (+) of Figure 6] has been obtained by Massari et al. (1978) for entirely different PG and PC species and by a much different technique (analysis of dye binding). Massari et al. sonified phospholipid samples with an immersed titanium tip in an EDTA-containing buffer.

Assuming that Mn^{2+} does induce an equilibrium distribution of phospholipids, our results indicate that PG disproportionates to the outer leaflet of SUV membranes at low PG content (Figure 6), despite the fact that PG and PC display nearly ideal lateral mixing at low PG content in both multilamellar vesicles and SUV (Lentz et al., 1982). Disproportionation of PG to the outer membrane leaflet is consistent with our previous proposal that phospholipids in a phosphatidylcholine matrix would tend toward the outer leaflet at low mole fractions due to the greater ability of this leaflet to accommodate an impurity in the PC matrix (Lentz et al., 1980). At higher mole fractions of PG, our data and those of Massari et al. indicate that head-group charge repulsion is not sufficient to favor an asymmetric distribution. It is interesting that the preference of PG for the outer membrane leaflet was found to be less than that of phosphatidylethanolamine in mixtures with phosphatidylcholine. Since phosphatidylethanolamine is uncharged at neutral pH, this implies that, even at low mole fraction, head-group repulsion is not as important a driving force for membrane asymmetry as are such factors as head-group size and probably acyl chain composition. This assumes, of course, that reported asymmetric distributions of phosphatidylethanolamine (Litman, 1973; Lentz & Litman, 1978) represent equilibrium arrangements.

In light of the catalytic nature of the role of Mn^{2+}, it is worthwhile speculating about the possible mechanism of the redistribution process. The sensitivity of redistribution of lipid and Mn^{2+} concentration indicates at least a bimolecular interaction between vesicle and ion. We have insufficient kinetic data to determine if a ternary (vesicle–ion–vesicle) interaction might by involved. The molecular nature of the ion–lipid complex allowing transbilayer migration of lipids while still maintaining a permeability barrier to Mn^{2+} is enigmatic. One can imagine an inverted micellar complex of the sort proposed to be involved in the process of membrane fusion (Cullis & DeKruijff, 1979). However, it is difficult to understand how such inverted micellar structures might be consistent with the observed lack of Mn^{2+} movement across the bilayer. It may be that a fluctuation in bilayer structure consistent with rapid transbilayer lipid migration occurs at the interface of two vesicles linked by a Mn^{2+} ion. Such close bilayer contact has been proposed to explain the "lipidic particles" observed in an egg PC/cardiolipin vesicle preparation upon addition of Ca^{2+} (Rand et al., 1981).

A unique feature of our current results is the specificity displayed by Mn^{2+} and Cd^{2+} in inducing transbilayer reorganization. While Ca^{2+} has been noted to interact specifically with phosphatidylserine (Portis et al., 1979), little precedent exists for the specific ion effects reported here. One published study has claimed to show Ca^{2+}-induced cardiolipin "flip-flop" in negatively charged vesicles (Gerritsen et al., 1980). However, this work failed to provide sufficient evidence to demonstrate transbilayer migration as opposed to other changes in vesicle morphology. Therefore, to the best of our knowledge, the results reported here represent the only clear demonstration of a membrane transbilayer reorganization induced by minor amounts of specific divalent metal ions. In this regard, there are at least two reports in the recent literature that provide evidence for specific effects of Mn^{2+} (and Cd^{2+}) on cellular membranes. One is the demonstrated requirement for Mn^{2+} or Cd^{2+} in the regeneration of active "tissue factor" from its purified apoprotein and phospholipids (Carson & Konigsberg, 1980). The other is a recent report of a Mn^{2+}-specific fusion process occurring in golgi fractions (Paiement et al., 1982; J. M. Bergeron, personal communication). These observations suggest that important functional consequences may result from specific ion–lipid interactions involving cellular divalent metal ions other than Ca^{2+}.

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Glycoprotein Topology on Intact Human Red Blood Cells Reevaluated by Cross-Linking following Amino Group Supplementation†

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ABSTRACT: Protein–protein interactions were studied at 0–4 °C with amino group specific cross-linkers on intact human erythrocytes after introducing free alkylamino groups into glycoproteins to overcome the scarcity of accessible amino groups. Amino group supplementation is based on the formation of aldehydes by either enzymatic or chemical oxidation. Subsequently, an imine is formed between aldehyde groups and 2-(4-aminophenyl)[1-^{14}C]ethylamine (arylalkyldiamine) in the presence of sodium cyanoborohydride ($NaCNBH_3$). The arylamino group of arylalkyldiamine forms imines at least 280 times more rapidly than the alkylamino group. This property leaves the majority of alkylamino groups free for subsequent cross-linking with bifunctional reagents. Amino group supplementation enhances the cross-linking probability of glycophorins in glycophorin-containing vesicles. When applied to intact human erythrocytes of any age, in conjunction with the cross-linker disuccinimidyl 3,3′-dithiobis(propionate), the glycoproteins, band 3 and glycophorins, did not undergo substantial cross-linking (less than 2% of the total label cross-linked). However, substantial cross-linking (20% of the total label cross-linked) of either glycoprotein was detected on spectrin-free vesicles [Lutz, H. U., Liu, S. C., & Palek, J. (1977) *J. Cell Biol. 73*, 548–560] that are devoid of cytoskeletal restraints. The inability to cross-link these proteins on intact cells is not due to a lack of accessible amino groups on the surface of these glycoproteins, because amino group supplementation enhanced their ability to form cross-links on intact cells and on spectrin-free vesicles by the same factor (2–3-fold). This and various controls suggest a monomeric arrangement of the exoplasmic portions of band 3 on *intact* erythrocytes.

Chemical cross-linking of memrane proteins has been studied extensively on isolated erythrocyte membranes (Steck, 1972; Ji, 1973; Wang & Richards, 1974; Staros et al., 1974; Lutz et al., 1977a). These investigations have generated a considerable knowledge of possible protein–protein interactions within the membrane, among them the existence of band 3 dimers (Wang & Richards, 1974; Nigg & Cherry, 1979) but an absence of interactions between glycophorins (Steck, 1972; Capaldi, 1973). However, only a limited number of experiments were carried out on intact cells (Wang & Richards, 1975). These studies revealed essentially the same results for intact cells and unsealed ghosts but were hampered by the fact that permeable reagents caused extensive cross-linking of cytoplasmic proteins and spectrin. Thus, concomitant cross-linking of band 3 could have been due to cytoplasmic cross-links or could occur passively as a consequence of cross-links within the cytoskeletal framework. Staros et al. (1981) recently applied an impermeable bifunctional cross-linking reagent to intact cells and could demonstrate band 3 cross-linking without simultaneously cross-linking cytoskeletal elements (Staros et al., 1981). This was taken as evidence for band 3 dimers on native membranes, although the conditions used to detect it (30 min at room temperature) clearly allowed long-range protein mobility to occur. Hence, these data cannot exclude the possibility that monomeric band 3 protein formed cross-linkable dimers in the course of diffusion-controlled collisions. Collisions of monomeric band 3 could occur at this elevated temperature in media that do not allow the cells to maintain their natural ATP content. Extensive ATP depletion is known to facilitate cross-linking of membrane proteins in intact cells as measured by endogenous SS-bridge formation at pH values close to the isoelectric point of the proteins involved (Liu et al., 1977). The glycoprotein topology of intact erythrocytes is further complicated by the fact that negligible extents of glycophorin cross-links were detectable (Ji, 1979). This was tentatively ascribed to an inaccessibility of endogenous amino groups (Marchesi & Furthmayr, 1976).

For these reasons we have developed a method to overcome the scarcity of accessible, endogenous amino groups by supplementing surface glycoproteins on intact cells with covalently

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