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Phase Behavior of Mixed Phosphatidylglycerol/Phosphatidylcholine Multilamellar and Unilamellar Vesicles†

Barry R. Lentz,* Dennis R. Alford, Mathias Hoechli, and Frederick A. Dombrose

ABSTRACT: The phase behavior of dipentadecanoylphosphatidylglycerol ($DC_{15}PG$)/dimyristoylphosphatidylcholine (DMPC) mixtures has been studied in both small, unilamellar vesicles and large, multilamellar vesicles. We have used both the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) and high-sensitivity differential scanning calorimetry to detect temperature-dependent changes in membrane structure. Electron microscopy has demonstrated different fracture face morphologies for large, multilamellar vesicles depending on sample composition and temperature. These data have been interpreted in terms of proposed phase diagrams for this lipid mixture. The shapes of the proposed phase diagrams have led us to conclude that DMPC and $DC_{15}PG$ mix freely in the plane of a lipid bilayer only at less than 50 mol % $DC_{15}PG$. At higher $DC_{15}PG$ content, the data have been interpreted as reflecting substantial compositional inhomogeneities in the plane of the bilayer, if not phase immiscibility, even in the fluid phase. In addition, small vesicles containing greater than 50 mol % $DC_{15}PG$ were unstable in the ordered phase and spontaneously converted to larger vesicles. Finally, the anisotropy of DPH fluorescence was found to be invariant with $DC_{15}PG$ content at temperatures just above the liquidus phase line in small, unilamellar vesicles. This demonstrated that inclusion of negatively charged phosphatidylglycerol does not noticeably affect the order within the acyl chain region of the bilayer, relative to phosphatidylcholine.

Phosphatidylglycerol is a major phospholipid class in plant and microbial membranes (Galliard, 1973) and is present in small amounts in mammalian membranes (McMurray, 1973). The ready availability and chemical stability of synthetic phosphatidylglycerols have made them attractive model compounds for studying the effects of acidic phospholipids on membrane structure and function. The pK_a is sufficiently low (2.9 at physiological ionic strength; Watts et al., 1978) that phosphatidylglycerol maintains a full negative charge at physiological pH. Several biophysical studies have focused on the phase behavior of pure synthetic phosphatidylglycerols (van Dijck et al., 1975; Jacobson & Papahadjopoulos, 1975; Ranck et al., 1977; Watts et al., 1978; Wohlgemuth et al., 1980; Cevc et al., 1980; Harlos & Eible, 1980). Physical techniques employed in these studies have included spin resonance spectroscopy, fluorescence polarization, X-ray diffraction, electron microscopy, and differential scanning calorimetry. Two recent papers report rapid scanning (300 and 560 °C/h) differential calorimetric measurements of phase separations in large, multilamellar vesicles (LMV)[1] composed of phosphatidylglycerol/phosphatidylcholine mixtures (van Dijck et al., 1978; Findlay & Barton, 1978). With the assumption that rapid scanning did not significantly perturb the system from equilibrium, these studies concluded that the glycerol and choline head groups allowed complete mixing of lipid species.

While most determinations of phase behavior have been made with LMV preparations, unilamellar vesicles are more appropriate model membranes for investigating interactions of proteins and lipids at a membrane surface. Small, unilamellar vesicle (SUV) preparations composed of synthetic phosphatidylglycerol/phosphatidylcholine mixtures have been used to study the Ca^{2+}-mediated binding of γ-carboxyglutamate-containing human prothrombin to negatively charged membrane surfaces (Dombrose et al., 1979). This is one of the several protein/lipid interactions that are thought to be crucial to the ability of phosphatidylglycerol/phospha-

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[1] Abbreviations: LMV, large, multilamellar vesicles; SUV, small, unilamellar vesicles; $DC_{15}PG$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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tidylcholine mixtures to enhance, in vitro, by several thousand fold the rate of blood coagulation (Jackson et al., 1974; Barton & Findlay, 1978). This clot-enhancing ability is thought to be provided, in vivo, by membranes derived from activated platelets (Bode et al., 1981). In order to determine the contribution of lipid phase structure to protein/lipid interactions relevant to clot-promoting activity, we have sought to establish the structural parameters of model membranes composed of mixtures of 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol ($DC_{15}PG$) and 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC). We have previously determined the trans-bilayer distribution of these lipids in SUV (Lentz et al., 1980a). In this report, we establish the phase behavior of mixtures of these lipids incorporated into LMV and SUV model membranes. In future studies, we will probe the effect of appropriate protein clotting factors on this phase behavior. An odd-chain synthetic phosphatidylglycerol ($DC_{15}PG$) has been chosen because its phase transition is just below the physiological temperature range. Use of unsaturated phosphatidylglycerol or dimyristoylphosphatidylglycerol would have defeated the purpose of producing observable phase separations near physiological temperatures. This allows protein binding studies to be carried out with fluid-phase lipid near physiological temperatures but below temperatures at which proteins will denature.

In this paper, we demonstrate that DMPC and $DC_{15}PG$ mix freely in the fluid and gel phases of both LMV and SUV having $DC_{15}PG$ contents less than 50 mol %. Above 50 mol % $DC_{15}PG$, our results suggest that phosphatidylglycerol-rich domains may exist in both the gel and fluid phases. This is a surprising result for mixtures of two lipids with similar acyl chains and conflicts with the conclusions of earlier studies. In addition, pure $DC_{15}PG$ or $DC_{15}PG$-rich SUV have been shown to undergo an irreversible change in vesicle morphology in the gel phase, requiring that care be taken to maintain them in the fluid phase before use. We conclude that mixed $DC_{15}PG$/DMPC SUV demonstrate a complex phase behavior, which justifies their careful characterization before using them as a model system for studies of protein/lipid and ion/lipid interactions.

Experimental Procedures

Materials. Dimyristoylphosphatidylcholine (DMPC) and the sodium salt of dipentadecanoylphosphatidylglycerol ($DC_{15}PG$) were purchased from Avanti Biochemical (Birmingham, AL). For removal of associated divalent cations, the commercial $DC_{15}PG$ was dissolved in 1/1 (v/v) chloroform/methanol to a concentration of 0.02 M and thrice extracted with an equal volume of an aqueous phase containing 0.1 M disodium ethylenediaminetetraacetate (EDTA; pH 7.5). The solvent was evaporated from the lower phase, and the lipid residue was twice recrystallized by adding acetone to a 1/1 (v/v) chloroform/methanol solution (Lentz et al., 1976). Flame ionization analysis on a Perkin-Elmer 305B atomic absorption spectrometer equipped with a calcium-specific detector revealed less than 2 mol of calcium remaining per 100 mol of lipid. Both lipids were judged better than 99% pure by thin-layer chromatography (Lentz et al., 1976) and were stored as previously described (Lentz et al., 1980a). [^{14}C]-DMPC was synthesized (Patel et al., 1979) from [1-^{14}C]-myristic acid (RPI Corp., Elk Grove, IL; lot 1076-178) and added to DMPC stocks. Lipid concentrations of stock solutions and vesicle preparations were established by phosphate determinations (Chen et al., 1956). Vesicular DMPC content was established by scintillation counting. All chemicals were reagent grade or better. Water was distilled from basic potassium permanganate and then redistilled from glass, bubbled with argon, and stored under argon at 4 °C. Zone-purified 1,6-diphenyl-1,3,5-hexatriene (DPH) was a gift from M. Shinitzky and Y. Barenholz.

Vesicles. LMV were prepared from quantitative mixtures of stock solutions of DMPC and $DC_{15}PG$. The organic solvent was removed under a stream of argon, and the lipid residue was further dried under high vacuum overnight. The dried sample, coated in a thin film on the wall of a round-bottom flask, was warmed to 40–45 °C, and an appropriate amount of warm buffer (100 mM NaCl, 10 mM Tris, and 0.02% sodium azide, pH 7.4) was added with vigorous vortexing for 1–2 min. Samples were gently swirled at 45–48 °C for at least 8 h before use. SUV suspensions were prepared in a different buffer (100 mM NaCl, 10 mM Tes, and 0.02% sodium azide, pH 7.4) by using a Heat Systems W350 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) equipped with a Heat Systems Cup-Horn (Barrow & Lentz, 1980) and then fractionated by ultracentrifugation as previously described (Lentz et al., 1980a). In some instances, 100 μM EDTA was included in the Tes buffer system. SUV preparations were always maintained above their phase transition temperature following sonication and were typically used within 2–14 h after ultracentrifugation.

Fluorescence Measurements. DPH was introduced into vesicle suspensions at 45 °C by injecting, with vigorous vortexing, a small volume (0.2–0.4 μL) of 2 mM DPH dissolved in tetrahydrofuran. The final dye:lipid ratio for most preparations was 1:500. Measurements of DPH fluorescence and fluorescence anisotropy were made in heating and cooling scans at rates of ±18 °C/h by using a T-system SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL). Details of the measurements as well as of calculation of the "microviscosity" and "microviscosity activation energy" parameters are given in the literature (Lentz et al., 1978, 1980b). The validity of these techniques as procedures for detecting phospholipid phase transitions has been established for a variety of lipid mixtures by comparison with calorimetric data (Lentz et al., 1976, 1978, 1980b).

Freeze–Fracture Electron Microscopy. Samples were slowly cooled (−6 °C/h) from 45 °C to freeze-quenching temperatures, equilibrated, and then rapidly jet frozen as previously described (Lentz et al., 1980, 1981). A modification of previous procedures was the use of copper sheets coated with Formvar (Ted Pella, Inc., Tustin, CA) to prepare the sample sandwich. This helped prevent fracturing along the sample/copper interface but slowed somewhat the freezing rate relative to our old procedure (Lentz et al., 1980b). Sephadex CM52 (Pharmacia, Piscataway, NJ) dispersed in silicon grease (Dow Corning high vacuum) was applied at the edges of the copper sheets to keep the sheets 20 μm apart as well as to help seal the sample from evaporation during the incubation. The well-defined and small gap between the copper sheets resulted in faster cooling rates relative to our previous procedure. This modification helped prevent both fracturing along the sample/copper interface as well as clustering of the charged liposomes on the copper surface. The net result of these modifications was to produce comparable freezing rates but improved fracturing relative to our previous procedures (Lentz et al., 1980b, 1981).

Calorimetry. A high-sensitivity scanning calorimeter of the differential heat flow type was specially constructed for biological calorimetry by Roger Hart (originally of Tronac, Inc., and currently of Hart Scientific, Orem, UT). This instrument is based on the design used by Suurkuusk et al. (1976) but with the improvements of increased sensitivity and the ability

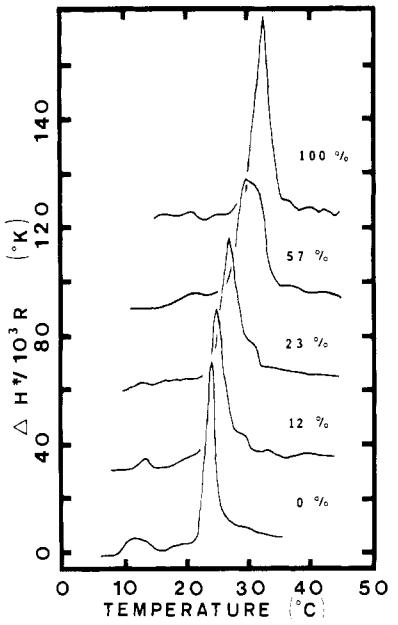

FIGURE 1: Temperature dependence of the DPH-derived microviscosity activation energy (ΔH^*) in large, multilamellar vesicles composed of several representative mixtures of DMPC and $DC_{15}PG$. The sample compositions are indicated next to each plot (mole percent of $DC_{15}PG$). Data for cooling scans are shown; heating scans were essentially identical. The 0% data are plotted according to the units of the ordinate, while successive curves are displaced upward by increments of 30 units each.

to cool as well as to heat. Data collection is under microprocessor control. Temperature accuracy is better than 0.05 °C, and base line noise is less than 0.02 μcal/s. As with any heat flow calorimeter, computer calculations based on a Newtonian heat flow model are necessary in order to obtain excess heat capacities independent of the dynamic response of the calorimeter. The success of these calculations has been verified with samples of known phase behavior (e.g., dipalmitoylphosphatidylcholine LMV). The details of these calculations as well as of calorimeter design will be published elsewhere (B. R. Lentz, K. W. Clubb, R. Hart, and R. L. Biltonen, unpublished).

Results

Phase Behavior of Large, Multilamellar Vesicles. In this section, we establish the phase behavior of LMV prepared from mixtures of DMPC and $DC_{15}PG$ and use this information to propose a temperature/composition phase diagram for these two-component membranes. For the most part, we have used changes in the fluorescence polarization of DPH to detect lipid phase transitions. Representative plots of the DPH-derived microviscosity activation energy (Lentz et al., 1978) vs. temperature are given in Figure 1. Only cooling scans are shown, since heating and cooling experiments gave essentially identical results except in the region of the pretransition. Cooling scans for mixtures of DMPC and $DC_{15}PG$ generally showed the pretransition at slightly lower temperatures than did heating scans, a phenomenon that we have described previously in the case of pure phosphatidylcholine (Lentz et al., 1978). In samples prepared from pure $DC_{15}PG$, a clearly defined pretransition was not detectable in either heating or cooling scans (see Figure 1). Even in $DC_{15}PG$/DMPC mixtures, the pretransition peak (see Figure 1) was considerably less apparent than in mixtures of synthetic phosphatidylcholines (Lentz et al., 1976). In order to test for the existence of a pretransition in a pure $DC_{15}PG$ sample, we performed high-sensitivity

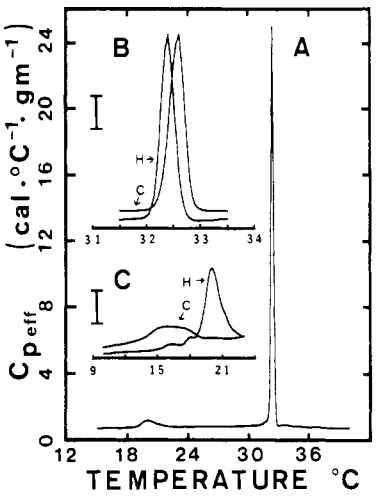

FIGURE 2: Temperature dependence of the effective heat capacity ($C_{p\,eff}$) of a $DC_{15}PG$ LMV sample of concentration 8.3 mM in 0.10 M NaCl, 0.01 M Tes, and 0.02% NaN_3 buffer, pH 7.4. Sample volume was 1.536 mL. The sample was first heated at a rate of +15.0 °C/h and then cooled at a rate of −16.8 °C/h. The entire heating scan is shown in curve A, which is plotted according to the ordinate scale. Heating (H) and cooling (C) scans are presented according to modified ordinate and abscissa scales for the main transition (curves B) and pretransition (curves C) regions. The ordinate scale marker for the B curves corresponds to 4.0 cal $°C^{-1}$ g^{-1} while that for the C curves corresponds to 0.2 cal $°C^{-1}$ g^{-1}.

differential scanning calorimetric experiments. The resulting heat capacity profiles are shown in Figure 2. The pretransition clearly is present in both but is more prominent in the heating scan (Figure 2A,C; T_m = 19.9 °C, ΔH_{ex} = 0.6 kcal/mol) than in the companion cooling scan (Figure 2C; T_m = 16.4 °C, ΔH_{ex} = 0.3 kcal/mol). The cooling measurement indicates a broader pretransition than does the heating scan, another parallel to the behavior of pure phosphatidylcholines (Lentz et al., 1978). The main phase transition of $DC_{15}PG$ LMV, on the other hand, is seen to be nearly identical in heating and cooling scans (Figure 2A,B; T_m = 32.4 °C, ΔH_{ex} = 5.9 kcal/mol for heating; T_m = 32.6 °C, ΔH_{ex} = 5.8 kcal/mol for cooling). The difference in T_m values (0.2 °C) is in the opposite direction of that expected for artifacts due to rapid heating or cooling rates and may represent the limit in accuracy of our computer calculations to correct for calorimeter response.

The delimiting temperatures of the phase transition fluorometrically and calorimetrically in $DC_{15}PG$/DMPC large, multilamellar vesicles are summarized in the temperature/composition diagram shown in Figure 4A. In the case of the pretransition, only the calorimetric heating data are shown, since the cooling transition was very broad due to kinetic effects (Lentz et al., 1978).

In order to further define the phase behavior of $DC_{15}PG$ and its mixtures with DMPC, we have characterized the fracture face morphologies of samples of varying composition which were jet frozen from a variety of temperatures. Characteristic morphologies are shown in Figure 3. Figure 3a–d illustrates the morphological changes associated with the phase behavior of pure $DC_{15}PG$. At high temperatures, a mottled (M) morphology was found (Figure 3a), analogous to that observed in pure phosphatidylcholine or sphingomyelin (Lentz et al., 1980b, 1981). In the region of the main phase transition, mixed morphologies were observed (rivuletted, R; banded, B; and jumbled, J; Figure 3b). Jumbled morphologies have been reported before in phosphatidylcholine and probably reflect fluid phase regions that have begun to crystallize into imperfect rivuletted structure during the very short but finite time required for freezing (Lentz et al., 1980b). The rivuletted

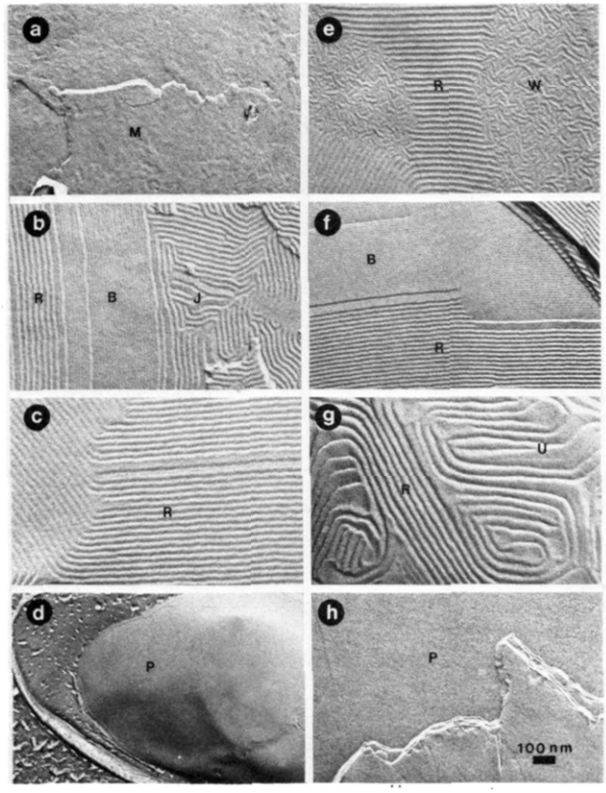

FIGURE 3: Electron micrographs of freeze–fracture replicas prepared from DMPC/$DC_{15}PG$ LMV quenched from several temperatures, magnification 33750×. Samples shown are pure $DC_{15}PG$ frozen from (a) 50, (b) 35, (c) 29, and (d) 13 °C and (e) 66 mol % $DC_{15}PG$ frozen from 34.4 °C, (f) 26 mol % frozen from 21 °C, (g) 26 mol % frozen from 16.5 °C, and (h) 63 mol % frozen from 10 °C. Fractured samples were platinum shadowed at an angle of 45°. Micrographs are shown with shadowing from below.

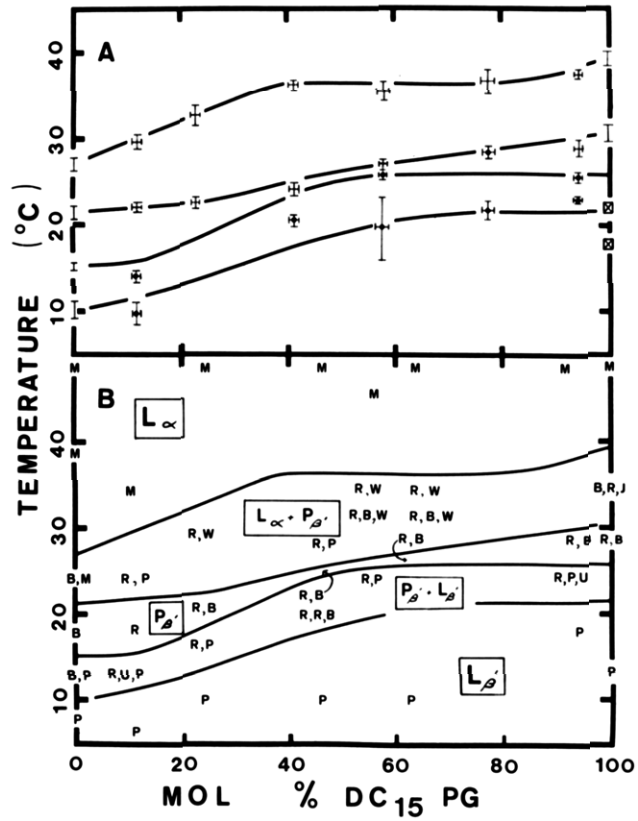

FIGURE 4: Temperature/composition diagram for $DC_{15}PG$/DMPC large, multilamellar vesicles. (A) Temperature extremes taken from DPH microviscosity activation energy data (e.g., Figure 1) are indicated by error bars for the main transition and by dotted error bars for the pretransition. In both cases, composite results from cooling and subsequent heating scans are presented; for the main transition, these agreed, while for the pretransition they did not (Lentz et al., 1978) and an average was used. Uncertainties in the temperatures (range of delimiting temperatures from heating and cooling scans) and composition (one standard deviation) are indicated by the vertical and horizontal bars, respectively. Calorimetric data were used to obtain the delimiting temperatures (crossed squares) for the pure $DC_{15}PG$ pretransition, since this transition was not clearly revealed by DPH fluorescence changes. (B) Summary of freeze–fracture electron microscopy results together with our interpretation in terms of a phase diagram for this lipid mixture. Observed morphologies are indicated by the following: P, planar; M, mottled; R, rivuletted; B, banded; J, jumbled; U, undulated; W, wormy.

and banded morphologies previously have been associated both with the $P_{\beta'}$[2] phase of pure phosphatidylcholines (Luna & McConnell, 1976; Lentz et al., 1980b) and with a similar phase observed in synthetic sphingomyelins (Lentz et al., 1981). A unique feature of the banded or rivuletted morphologies observed in phosphatidylglycerol multilayers is the reduced orientational correlation of structures in different bilayers (e.g., see Figure 3f) relative to that found in pure phosphatidylcholine or sphingomyelin systems (Lentz et al., 1980b, 1981). In the latter systems, rivulettes or bands in adjacent bilayers are more likely to be observed running parallel to one another. The observed reduced interbilayer correlation probably reflects the increased thickness of the water layer between lipid bilayers in multilamellar structures composed of charged lipids (Atkinson et al., 1974). The observations of pure rivuletted or banded morphologies (Janiak et al., 1976; Luna & McConnell, 1976) at temperatures immediately below the main phase transition (Figure 3c) and of a planar (P) morphology at even lower temperatures (Figure 3d) confirm our calorimetric observation of a pretransition in pure $DC_{15}PG$. It is noteworthy that LMV preparations rich in $DC_{15}PG$ contained small vesicles with only one to three widely separated lamellae, as judged by freeze–fracture electron micrographs (e.g., see Figure 3d). This is in contrast to the behavior of neutral phospholipids which, under similar circumstances, form very large, multilamellar structures. We have noted also that LMV rich in $DC_{15}PG$ scatter considerably less light than their pure neutral phospholipid counterparts. This could reflect the fact that widely spaced bilayers would be expected to result in less dramatic variations of refractive index with distance than would the closely spaced bilayers of phosphatidylcholine LMV.

In $DC_{15}PG$/DMPC mixtures, we observed a variety of fracture face morphologies, alone or in pairs, depending on the lipid composition and temperature from which the samples were frozen. Among the morphologies seen were "wormy" (W, Figure 3e), reflecting fluid phase regions partially crystallized into rivuletted structure (Lentz et al., 1980b), and "undulated" (U, Figure 3g). The latter morphology was uniquely associated with the pretransition in the phosphatidylglycerol-containing membranes studied here. The morphologies observed for a variety of compositions are summarized in the temperature/composition diagram in Figure 4B and interpreted there in terms of a phase diagram for $DC_{15}PG$/DMPC mixtures. Phase assignments were made by correlating the phase behavior and observed morphologies of different phases with observations for pure phosphatidylcholines (Janiak et al., 1976; Luna & McConnell, 1976; Lentz et al., 1980b).

[2] Lipid phase nomenclature is after Tardieu et al. (1973): L_α, lamellar, disordered acyl chains; $L_{\beta'}$, lamellar, ordered acyl chains extended at an acute angle to the bilayer normal; $P_{\beta'}$, pleated, ordered acyl chains extended at an acute angle to the bilayer normal.

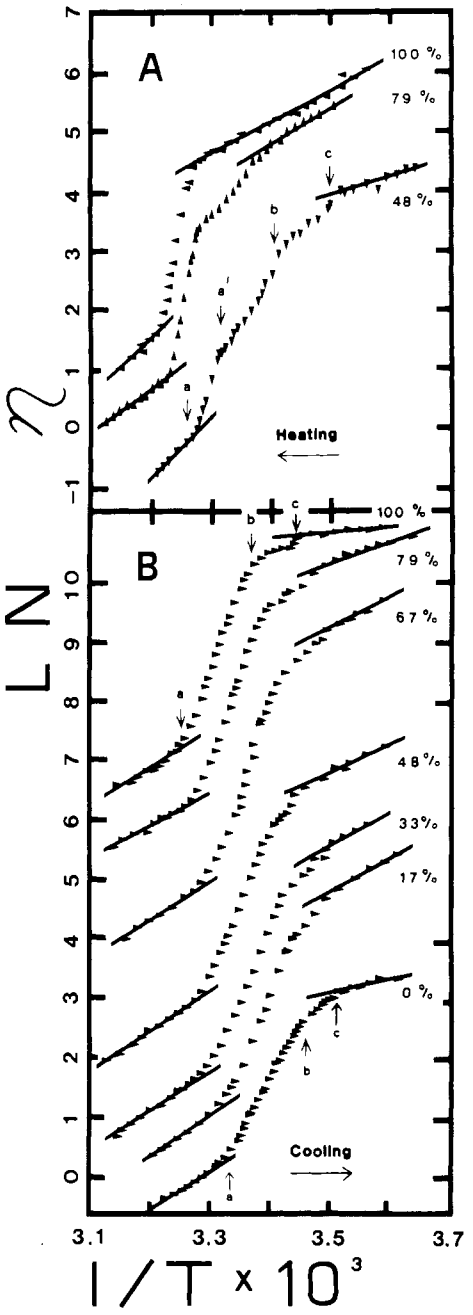

FIGURE 5: Arrhenius plots of DPH microviscosity for several mixtures of $DC_{15}PG$ (content shown) and DMPC incorporated into small, unilamellar vesicles. Data were collected in heating (A) and cooling (B) scans at rates of ±18 °C/h. Cooling scans were performed prior to heating scans. Arrows indicating break temperatures are discussed in the text. The lowermost data sets in both A and B are plotted according to the ordinate, while subsequent data sets are displaced upward by increments of 1 unit.

Phase Behavior of Small Vesicles. In analogy to the LMV studies, we have used changes in the fluorescence of DPH to follow temperature-dependent phase changes in $DC_{15}PG$/DMPC SUV. Unlike the LMV system, SUV preparations displayed irreversible phase behavior, a phenomenon which was explored in some detail. Figure 5 shows Arrhenius plots of the microviscosity parameter, which is derived from the DPH fluorescence anisotropy. Deviations of these plots from linearity have been well established as indicating a membrane phase change (Suurkuusk et al., 1976; Lentz et al., 1976). As in previous studies of mixed-lipid SUV (Lentz et al., 1976, 1981), the phase transition was indicated by a broad change in DPH microviscosity (arrows indicate delimiting temperatures at points a and c in Figure 5B) which could be subdivided further into two regions of relatively rapid (a to b) and slow change (b to c). Figure 5B displays cooling scans. For low content of $DC_{15}PG$, heating scans were essentially identical with cooling scans and are not shown. This was not the case above 50 mol % $DC_{15}PG$, however. In heating scans performed on samples containing greater than 50 mol % $DC_{15}PG$, we observed abrupt decreases in microviscosity at temperatures above the broad microviscosity change seen in cooling scans. This phenomenon is illustrated by comparing the cooling (Figure 5B) and heating (Figure 5A) scans for 48, 79, and 100 mol % $DC_{15}PG$/DMPC vesicles. The region of abrupt change is delimited by the symbols a and a′ in Figure 5A. The relative magnitude of the abrupt change increased with $DC_{15}PG$ content until, for pure $DC_{15}PG$, it was the sole change observed on heating (Figure 5A). Samples containing greater than 50 mol % $DC_{15}PG$ and displaying such behavior did not display obvious flocculation below the phase transition. The intensity of 366-nm light scattered at 90 °C by pure $DC_{15}PG$ vesicles was observed to increase slightly upon cooling, but no substantial increase was associated with passage through the phase transition.

Electron microscopy was utilized to test for structural changes in SUV taken below the phase transition. Freeze–fracture electron micrographs showed that a sample of $DC_{15}PG$ maintained at 45 °C and frozen from 50 °C consisted of unilamellar vesicles with mass-weighted, mean diameters of 220–270 Å. The same sample maintained for 2.5 h at 5 °C and then frozen from 50 °C showed dual peaks in the mass-weighted distribution of vesicle sizes. A fraction of the lipid was observed to have diameters of 180–330 Å, while the majority of the remaining lipid appeared in vesicles of diameters from 340 to 800 Å. Entirely analogous results were obtained with a $DC_{15}PG$ SUV sample prepared and maintained at 5 °C in the presence of 25 μM Na_2EDTA. This demonstrates that multivalent metal ion contamination was not responsible for vesicle morphology change below the order/disorder phase transition. The electron microscopic data demonstrate clearly that SUV containing greater than 50 mol % $DC_{15}PG$ are unstable at temperatures at which their acyl chains are in an ordered configuration.[3]

Despite the history-dependent behavior of $DC_{15}PG$/DMPC SUV stored below their phase transition, initial cooling scans performed on samples prepared in the presence or absence of EDTA and maintained above their phase transition were always reproducible. It is this aspect of SUV phase behavior that is relevant to our ultimate purpose of using them in the near-physiological range as a model system to explore the involvement of negatively charged phospholipid surfaces in blood coagulation. We have summarized in Figure 6 the delimiting temperatures of phase transition peaks obtained from cooling (and some heating) scans of $DC_{15}PG$/DMPC SUV of various compositions. Because of the clear evidence for irreversibility at high $DC_{15}PG$ content, this tempera-

[3] The reason for the inequality of heating and cooling scans appeared to be only in part the observed change in vesicle structure upon storage of SUV below their phase transition. When a pure $DC_{15}PG$ SUV preparation was (1) cooled, then (2) heated, and (3) finally cooled again, the final (cooling) phase behavior was not equivalent to that observed in the second (heating) experiment. The phase transition observed on heating was sharper and at somewhat higher temperature than that observed in the subsequent cooling scan (B. R. Lentz and D. R. Alford, unpublished experiments). Indeed, the phase transition in the heating scan occurred at a slightly higher temperature than did the phase transition in pure $DC_{15}PG$ LMV. We are currently seeking an explanation for these results in terms of possible metastable states occurring in the ordered phase of $DC_{15}PG$-rich samples.

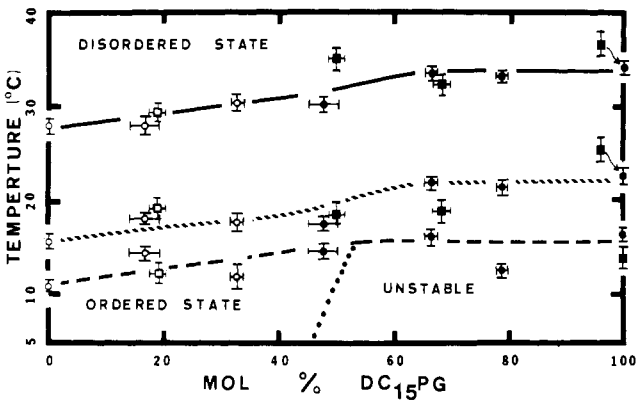

FIGURE 6: Temperature/composition diagram summarizing the phase behavior of small, unilamellar vesicles composed of mixtures of $DC_{15}PG$ and DMPC. The delimiting temperatures were obtained from Arrhenius plots such as those in Figure 5. Samples were prepared both in the presence (squares) and in the absence (circles) of EDTA. Data were obtained from both heating and cooling scans when these agreed (○, □) or from cooling scans when marked irreversibility was evident (●, ■). The upper line (—) was determined by breaks of the type labeled "a" in Figure 5, the middle line (- - -) by breaks of the type "b", and the lower line (– –) by breaks of the type "c". The ordered-phase region labeled "unstable" is that in which small vesicles were found to fuse into larger vesicle species, as determined by freeze–fracture electron microscopy (see text).

ture/composition diagram cannot be interpreted as an equilibrium phase diagram. However, it provides an overall picture that will be a useful tool in our discussion of SUV phase behavior and in future studies utilizing $DC_{15}PG$/DMPC SUV as a model system.

Discussion

We have sought in this study to establish the equilibrium phase behavior of both LMV and SUV composed of $DC_{15}PG$/DMPC mixtures. Fluorescence and calorimetric data were collected in both heating and cooling scans at slow scan rates (14–18 °C/h) to approach equilibrium conditions. Our purpose was 2-fold: first, to determine the ability of a neutral and a charged phospholipid to mix in the plane of both uncurved and highly curved membranes; second, to establish the usefulness of this lipid mixture for studies of ion/lipid interactions and of the role of negatively charged phospholipid surfaces in blood coagulation.

The ability of $DC_{15}PG$ and DMPC to mix in the plane of a fluid or gel phase bilayer is indicated by the shape of the phase diagrams in Figures 4 and 6. In both SUV and LMV preparations, the liquidus phase line (uppermost phase line) was found to be nearly horizontal in the region of 40 or 50 to 80 or 90 mol % $DC_{15}PG$. If this portion of the liquidus line could be unambiguously shown to be horizontal, it would be prima facie evidence for two coexisting fluid phases in the region of the phase diagram just above the horizontal line, as required by the phase rule (Gordon, 1968). However, we note that a phase line may be *close* to horizontal without the requirement of two coexisting phases occurring in the regions on either side of the line. Since the precision of our data is not sufficient to be *certain* that an isothermal phase line exists over a range of high $DC_{15}PG$ compositions, we may not conclude with certainty that two fluid phases coexist in $DC_{15}PG$-rich LMV or SUV. However, Von Dreele (1978) has presented a statistical thermodynamic treatment allowing one to interpret phase diagrams in terms of a nonideality parameter. In this treatment, a nearly isothermal phase line implies a large deviation from ideal mixing of the two lipid components in the phase adjacent to the phase line, and therefore a large nonideality parameter. Physically, this means that certain microscopic arrangements of molecules will be sufficiently energetically favorable as to make them much more probable than a random (i.e., ideal) distribution. This should result in substantial fluctuations away from a random distribution of lipids in the bilayer, i.e., in microdomains. The fact that the upper limit of the main phase transition in $DC_{15}PG$/DMPC mixtures was nearly the upper limit of the transition of pure $DC_{15}PG$ (Figures 4A and 6) would imply that microdomains containing ca. 80–90 mol % $DC_{15}PG$ are highly probable in the fluid phase of these mixed bilayers. Therefore, whether interpreted in terms of coexisting phases or in terms of fluctuating domains at equilibrium, our experimental results suggest that $DC_{15}PG$ and DMPC do not mix randomly in the fluid phase at $DC_{15}PG$ to DMPC ratios greater than one to one. This is an especially interesting result since this is the only binary mixture of similar chain length lipids shown to display such nonideality in the fluid phase. It is particularly surprising that the favored domains implied by our phase diagrams are rich in $DC_{15}PG$, since the charged head groups of this lipid would be expected to repel each other. Our data suggest the existence of interactions between phosphatidylglycerol head groups sufficiently favorable to overcome electrostatic repulsions, at least in a matrix containing a small amount of phosphatidylcholine and at moderately high ionic strength. By contrast, a similar study of a synthetic phosphatidylserine/phosphatidylcholine mixture gave no evidence for fluid phase inhomogeneity (Luna & McConnell, 1977). However, the delimiting temperatures summarized in Figures 4A and 6 were reproducibly obtained in duplicate preparations in the presence and absence of EDTA. Therefore, the isothermal portions of the liquidus lines in these figures are real and furthermore are not an anomaly due to divalent metal ion effects. We must conclude that $DC_{15}PG$ and DMPC are not randomly mixed in the plane of the membranes we have studied. Unfortunately, this conclusion disagrees with a previous report (Findlay & Barton, 1978) that phosphatidylglycerol and phosphatidylcholine species with acyl chains differing by two carbons were "highly miscible". However, this report was based on thermal analysis scans obtained at 560 °C/h on samples of relatively few compositions. The fluid phase inhomogeneities we describe are evident only by considering the complete phase diagram obtained under conditions approaching equilibrium.

In addition to information about the liquidus phase, our phase diagrams provide important information about the ordered phases of pure $DC_{15}PG$ and $DC_{15}PG$/DMPC mixtures. First, for large, multilamellar vesicles, our results confirm by high-sensitivity calorimetry the existence of a pretransition in pure disaturated phosphatidylglycerols. This transition had been reported previously by some (Jacobson & Papahadjopoulos, 1975; Findlay & Barton, 1978; Watts et al., 1978; Wohlgemuth et al., 1980) but not other investigators (van Dijck et al., 1975, 1978; Ranck et al., 1977). Our freeze–fracture electron micrographs (Figure 3) clearly establish the existence of such a transition both in pure $DC_{15}PG$ and in $DC_{15}PC$/DMPC mixtures. The properties of this transition (morphologies of gel phases, hysteresis) as well as its continuity between pure $DC_{15}PG$ and DMPC (Figure 4) suggest it to be essentially the same as the $P_{\beta'}$ to $L_{\beta'}$ transition observed in pure phosphatidylcholines (Janiak et al., 1976). Second, at greater than 1/1 $DC_{15}PG$/DMPC ratios, SUV preparations were found to be unstable in the ordered phase. Unlike phosphatidylethanolamine/phosphatidylcholine SUV preparations (Lentz & Litman, 1978), phosphatidylglycerol-containing

vesicles did not demonstrate a large increase in light scattering on being cooled into the unstable region of the phase diagram. However, vesicle size was rapidly and irreversibly altered by this procedure. The origin of this dramatic instability in the ordered phase is not known, but it is similar to the somewhat less rapid vesicle fusion that occurs upon storage of phosphatidylcholine SUV below the phase transition (Suurkuusk et al., 1976; Schullery et al., 1980). We note that 50 mol % dioleoylphosphatidylglycerol/dioleoylphosphatidylcholine SUV have been reported (Dombrose et al., 1979) to show no change in size distribution over a period of several months when stored at 4–6 °C, a temperature above the phase transitions of these lipids. Finally, for both LMV and SUV, our results give no indication of immiscible ordered phases, such as detected in cholesterol/phosphatidylcholine (Lentz et al., 1980b) and sphingomyelin/phosphatidylcholine (Lentz et al., 1981) mixtures. Nonetheless, there is in our results some indication of imperfect mixing of the two lipids in the gel phases at greater than 50 mol % $DC_{15}PG$, in analogy to the situation described in the fluid phase. The indications for this nonideality are the unstable region observed in the SUV phase diagram and the nearly horizontal phase lines bordering the $P_{\beta'} + L_{\beta'}$ region of the LMV phase diagram.

It is useful to compare the properties of the main phase transition of pure $DC_{15}PG$ as detected by calorimetric and fluorometric measurements. Comparison of the data in Figures 1 and 2 shows the calorimetric peak positions (T_m = 32.4 and 32.6 °C for heating and cooling, respectively) to agree well with the fluorometric peak positions (T_m = 32.6 and 32.4 °C for heating and cooling, respectively). However, DPH reports the main phase transition to be much broader than observed by differential scanning calorimetry (cf. Figures 1 and 2), as noted previously for pure phosphatidylcholine vesicles (Lentz et al., 1978). Our previous studies (Lentz et al., 1978) suggest that this broadening reflects sensitivity of the probe to a microscopic environment rather than perturbation of the membrane phase behavior by DPH. Calorimetric measurements reflect the melting of phospholipid acyl chains in fairly large cooperative units and thus yield narrow thermograms. In contrast, measurements sensitive to fluctuations in smaller regions, such as light scattering (Tsong & Kanehisa, 1977), probe fluorescence (Lentz et al., 1976; Sklar et al., 1977), and Raman scattering (Yellin & Levin, 1977), produce thermograms that are broader than those observed calorimetrically. With this in mind, the correspondence between the calorimetric and fluorometric peak positions extends the usefulness of DPH as a probe of membrane phase transitions to the charged lipid systems studied here.

The use of DPH fluorescence polarization changes to detect membrane phase transitions has the benefit that one obtains simultaneously a measure of order within the hydrophobic region of the membrane bilayer. It is of interest to ask whether the ratio of charged to neutral lipids affects the interior "fluidity" as measured by the extent of DPH motion. At 45 °C (in the fluid phase for all mixtures studied), we have found bilayer acyl chain order (as measured by DPH fluorescence anisotropy) to increase slightly with increased $DC_{15}PG$ content in SUV. However, when examined at 4 °C above the liquidus line in Figure 6, DPH fluorescence anisotropy was independent of phosphatidylglycerol content. At a constant interval below the solidus line, a similar result was obtained. We observed the same phenomenon in previous studies of mixtures of phosphatidylcholines having different acyl chain lengths (Lentz et al., 1976) and concluded that acyl chain order was dependent only on the relation of the bilayer temperature to its order/disorder phase transition. We conclude from the current observations that this situation is not altered by the presence of the negatively charged phosphorylglycerol head group as a component of the bilayer. We have observed also that the phase transition temperatures of dipentadecanoylphosphatidylglycerol and -phosphatidylcholine are indistinguishable (K. W. Clubb, R. A. Parente, and B. R. Lentz, unpublished experiments), adding further credence to the conclusion that the phosphorylglycerol head group does not alter acyl chain order, relative to phosphorylcholine.

We have noted the tendency of mixtures rich in $DC_{15}PG$ to form smaller LMV with fewer and more widely spaced lamellae than is common for neutral phospholipids. The observation of smaller vesicle sizes for pure phosphatidylglycerol LMV has been made previously by Jacobson & Papahadjopoulos (1975). We have also noticed that the orientation of rivuletted and banded structures in freeze–fracture replicas did not correlate as well between lamellae in $DC_{15}PG$-rich membranes as in neutral phospholipid samples. Both of these observations may be related to repulsion between neighboring negatively charged bilayers, especially at relatively low ionic strength. This repulsion should make structures with many closely spaced lamellae unfavorable and prevent the close approach necessary for coordinated head-group interactions between bilayers. This could explain the small enthalpy observed for the pretransition in $DC_{15}PG$ LMV (0.3–0.6 kcal/mol) relative to that for pure dipentadecanoylphosphatidylcholine LMV (1.0–1.4 kcal/mol; K. W. Clubb, R. A. Parente, and B. R. Lentz, unpublished experiments). We have observed a similarly reduced pretransition enthalpy in large, unilamellar vesicles that necessarily lack interbilayer interactions (R. A. Parente and B. R. Lentz, unpublished experiments).

Our second major purpose in this study was to determine the usefulness of $DC_{15}PG$/DMPC mixed vesicles for studies of ion/lipid interactions and of the role of negatively charged phospholipid surfaces in blood coagulation. In this regard, we have shown that $DC_{15}PG$/DMPC SUV membranes are fluid at physiological temperatures, which should make them useful model systems for studying the membrane interactions of polypeptide clotting factors. Previously, we had determined the transbilayer distribution of phosphatidylglycerol in these membranes (Lentz et al., 1980). We have demonstrated recently that this distribution is also sensitive to small concentrations of certain divalent cations (B. R. Lentz, C. S. Madden, and D. R. Alford, unpublished experiments). Work is now under way to determine the effects that interaction with Ca^{2+} and several human clotting factors will have on lateral phase separation and transbilayer lipid distribution in this model membrane system.

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