Segrest, J. P., Kahane, I., Jackson, R. L., and Marchesi, V. T. (1973), *Arch. Biochem. Biophys. 155*, 167.
Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G., and Ballantyne, D. L. (1971), *Biochemistry 10*, 4912.
Spatz, L., and Strittmatter, P. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68*, 1042.
Strauss, A. W., Donohue, A. M., Bennet, C. D., Rodkey, J. A., and Alberts, A. W. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74*, 1358.
Svasti, J., and Milstein, C. (1972), *Biochem. J. 126*, 837.
Thibodeau, S. N., Gagnon, J., and Palmiter, R. (1977), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 656.
Tonegawa, S., Brack, C., Hozumi, N., and Schuller, R. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74*, 3518.
Weigert, M. G., Cesari, I. M., Yonkovich, S. J., and Cohn, M. (1970), *Nature (London) 228*, 1045.
Wolf, O., Zemell, R., Burstein, Y., and Schechter, I. (1977), *Biochem. Biophys. Res. Commun., 78*, 1383.
Wu, T. T., and Kabat, E. A. (1970), *J. Exp. Med. 132*, 211.
Zemell, R., Burstein, Y., and Schechter, I. (1977), *Isr. J. Med. Sci. 13*, 935.

Phase Behavior of Synthetic Phosphatidylglycerols and Binary Mixtures with Phosphatidylcholines in the Presence and Absence of Calcium Ions†

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ABSTRACT: Using differential thermal analysis, scanning calorimetry and light scattering, transition temperatures and enthalpy data for the gel to liquid crystalline phase transitions of five synthetic phosphatidylglycerol sodium salts (PG-Na^+) were measured. The values obtained were almost identical with literature values for the corresponding phosphatidylcholines (PC). However, transition temperatures for the fully protonated forms of the saturated phosphatidylglycerols (PG-H^+) were approximately 20 °C higher. For binary mixtures of PG-Na^+ and PC in which the acyl chains of the two species were identical, the width of the thermal transition for the phase change was not appreciably greater than that observed with either of the two components alone. In contrast, mixing of PG-Na^+ and PC with different chain lengths increased the transition width. In the presence of Ca^{2+}, narrow transitions were also observed with mixtures of PG and PC when the chain length of the PG-Ca^{2+} was equal to or two carbons shorter than the PC but the transition width was clearly increased when the chain length of the PG-Ca^{2+} was two carbons longer than the PC. Mixing lipids with greater differences in chain length or mixing saturated lipids with unsaturated lipids in the presence of Ca^{2+} produced two minima in the thermograms, clearly indicative of phase separation. In sum, these results provide evidence for a high degree of miscibility of the phosphoglycerol and phosphocholine head groups, either in the presence or absence of Ca^{2+}, such that the characteristic phase behavior of each mixture is determined primarily by differences in the hydrocarbon chain structure.

Recently, the improved availability of synthetic phosphatidylglycerols (PG) has stimulated examination of the physical properties of the hydrated lipids (Tocanne et al., 1974; Verkleij et al., 1974; Ververgaert et al., 1975; Van Dijck et al., 1975; Jacobson & Papahadjopoulos, 1975; Papahadjopoulos et al., 1973, 1976; Jackson et al., 1974). These studies have utilized mainly differential scanning calorimetry (DSC)[1] and freeze fracture electron microscopy. Complex effects of divalent metal ions on the phase structures formed by dilauroylphosphatidylglycerol (DLPG), dimyristoylphosphatidylglycerol (DMPG), and dipalmitoylphosphatidylglycerol (DPPG) have been reported. The range of existence of the phase structures varied with the metal ion and acyl chain structure of the lipid.

Since biological membrane systems invariably contain mixtures of acidic and zwitterionic phospholipids, it was thought to be useful to characterize the behavior of binary mixtures of phosphatidylglycerols and phosphatidylcholines (PC) in the presence and absence of Ca^{2+} ions. Such mixtures have not previously been systematically investigated as a function of acyl chain length and polar head group composition. We have found that the thermotropic mesomorphism ranges from nearly ideal mixing behavior of the acidic and zwitterionic components at all concentrations examined to distinct phase separations with limited mutual miscibility of the two lipid components. The complex metastable behavior characteristic of the pure PG-Ca^{2+} or PG-Mg^{2+} is not observed with the mixtures.

Materials and Methods

Inorganic chemicals and reagents used in preparation of buffers were obtained from J. T. Baker Chemical Co., or Fisher Scientific Co., and were ACS reagent grade or better. All or-

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[1] Abbreviations used: DLPG, dilauroylphosphatidylglycerol (1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol); DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPS, dipalmitoylphosphatidylserine; DTA, differential thermal analysis; DSC, differential scanning calorimetry.

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ganic solvents were glass distilled. Water was deionized and glass distilled. Silicic acid used for column chromatography was "Unisil" (200–325 mesh) from Clarkson Chemical Co., Williamsport, Pa., or "Sorbsil" from Joseph Crossfield and Sons, Warrington, U.K. Carboxymethylcellulose was Whatman CM52 prepared as described by Comfurius & Zwaal (1977). Plates used in thin-layer chromatography were obtained, precoated with Silica Gel 60 F254, from Brinkmann Instruments, Rexdale, Ontario. α-L-Lecithin (β,γ-dilauryl) (lot 400203) and α-L-lecithin (β,γ-distearoyl) (lot 400204) were obtained from Calbiochem, San Diego, Calif. α-L-Phosphatidylcholine-dipalmitoyl and α-L-phosphatidylcholine-dimyristoyl were obtained from Sigma Chemical Co., St. Louis, Mo. The dioleoylphosphatidylcholine was that described in an earlier study (Barton & Gunstone, 1975). All synthetic phosphatidylcholines used were >98% pure as determined by thin-layer chromatography and gas–liquid chromatography of their fatty acid methyl esters. Phospholipase D from cabbage leaves was obtained from Sigma. The enzyme was supplied as a desiccated powder and was made up to the required concentration in 0.1 M sodium acetate buffer at pH 5.6.

Phospholipid phosphorus was determined by the method of King (1932). For synthetic lipids, calculations were based on the anhydrous molecular weight. Fatty acid methyl esters were prepared and isolated by the method of Brockerhoff (1965). The methyl esters were analyzed using a Hewlett-Packard 5700A gas chromatograph equipped with a diethylene glycol succinate column. Quantitation of peaks was by Hewlett-Packard 3370B integrator. Peaks were identified by reference to standards from Applied Science Laboratories, State College, Pa.

The method used to synthesize the diacylphosphatidylglycerols from the corresponding diacylphosphatidylcholines was essentially that of Dawson (1967). After termination of the reaction and extraction according to Bligh & Dyer (1959), considerable variation was observed in the distribution of the five different PG compounds among the organic, aqueous, and interfacial material. These variations necessitated close monitoring of the extraction procedures by thin-layer chromatography and phosphorus determinations to minimize loss of product. The lipids were purified by chromatography on silicic acid or carboxymethylcellulose using chloroform–methanol mixtures for elution (Comfurius & Zwaal, 1977). The Na^+ salts were prepared by titration of the Bligh & Dyer monophase to pH 9.5 with NaOH and the protonated phosphatidylglycerols by titration to pH 2.0 with HCl.

Differential Thermal Analysis (DTA). Aliquots of stock lipid solution were dried under N_2 to remove all visible solvent and then stored under vacuum at room temperature for 10 to 12 h to remove final traces of solvent. The dried lipid, usually 2 to 3 mg, was placed in a 2-mm diameter capillary tube and the aqueous component added with a Hamilton microliter syringe. The tube was then sealed and the lipid sample hydrated by the method of Chapman et al. (1967). When mixtures of lipids were required, the lipids were combined in the required molar amounts in $CHCl_3$ before drying under N_2. Thermal analysis was carried out with a Du Pont 900 thermal analyzer using the micro heating block. Rate of heating and cooling was approximately 7 °C/min. Anhydrous N_2, cooled by passage through a copper coil submerged in liquid N_2, was used for cooling. Thermograms were recorded on graph paper corrected for nonlinearity of the Chromel-Alumel thermocouples.

Differential Scanning Calorimetry (DSC). Most determinations were carried out using the Du Pont 900 thermal analyzer with DSC attachment. The samples were prepared by drying to constant weight in aluminum pans, rehydrating with known volumes of buffer and sealing the pans by crimping on aluminum lids. These samples were equilibrated for one or two days before scanning.

TABLE I: Thermodynamic Data for 1,2-Diacyl-*sn*-3-phosphoglycerols (PG) and 1,2-Diacyl-*sn*-3-phosphocholines (PC).

Lipid chains	PG-H^+ [a] (°C)	PG-Na^+ [b] T_c (°C)	PG-Na^+ [b] ΔH (kcal/mol)	PC [d] T_c (°C)	PC [d] ΔH (kcal/mol)
$C_{12:0}$	30	~4		~0	
$C_{14:0}$	47	23.7	6.94[c]	23	6.64
$C_{16:0}$	64.5	41.5	8.9	41	8.66
$C_{18:0}$	75	54.5	10.5	58[e]	10.67
$C_{18:1}$		−18		−22	7.6

[a] Phosphatidylglycerols extracted from the Bligh and Dyer monophasic solvent, pH 2.0, were dispersed in 0.01 N HCl. [b] Phosphatidylglycerols extracted from the Bligh and Dyer monophasic solvent, pH 9.5, were dispersed in Tris-HCl or glycine-NaOH, pH 9.5, 0.15 M NaCl. [c] Value determined in a high-sensitivity calorimeter (Privalov calorimeter) by Dr. J. M. Sturtevant. [d] Values from Phillips et al. (1970). [e] A value of 55 °C was obtained in the present study.

Light Scattering Experiments. The light scattering experiments were performed in a Perkin-Elmer MPF-4 fluorescence spectrophotometer with a circulating water bath providing the heating/cooling modes. The temperature of the sample was monitored by a thermocouple in direct contact with the sample solution and equipped with digital readout. The transition widths determined by the light scattering experiments were calculated graphically and represent the limits from 10% to 90% of the detectable intensity ($I_{90°}$) change for comparison with calorimetric data (Hinz & Sturtevant, 1972).

Sonication of Lipids. Sonication of lipid samples was done with a Bronwill Biosonik IV ultrasonicator with a titanium tip. Low intensity sonication was used to disperse lipids in aqueous solutions. For short bursts of sonication (0–60 s) the samples were at room temperature. If prolonged sonication was required, the samples were placed in an ice bath or in a water-cooled cell. The dispersions were then briefly centrifuged to remove coarse particles and the phospholipid content of the supernatant was directly determined.

Results

DTA and DSC of the Sodium Salts of Synthetic Phosphatidylglycerols. The thermograms of the pure synthetic phosphatidylglycerols (PG-Na^+) were virtually identical with those obtained for the homologous phosphatidylcholines (PC). The midpoint temperature of the thermal transition (DTA) and the enthalpy change (DSC) for each lipid is shown in Table I. With each of the saturated phosphatidylglycerols, a pretransition endotherm was observed that was qualitatively similar to those observed with the pure saturated phosphatidylcholines. Pretransition endotherms were not observed with either of the dioleoyl phospholipids.

DTA of Protonated Phosphatidylglycerols. The fully protonated phosphatidylglycerols (PG-H^+) also exhibited sharp thermal transitions at temperatures about 20 °C higher than those of the corresponding PG-Na^+ (Table I). Pretransition endotherms were also observed. Under conditions in which the lipids were partially protonated and partially in the salt form (pH 3 to pH 8), broad or multiple transitions were observed, similar to previously reported data for phosphatidylglycerols (Verkleij et al., 1974).

DTA of Mixtures of Phosphatidylglycerol Sodium Salts and Phosphatidylcholines with Identical Acyl Chains. Lipid

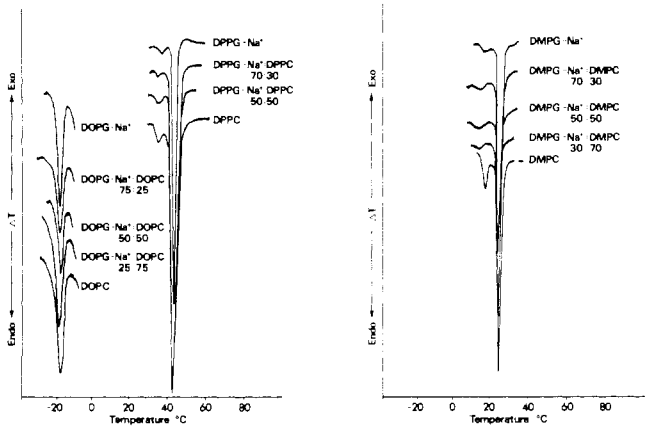

FIGURE 1: Differential thermal analysis of PG-Na^+:PC mixtures in which the two lipid species had identical acyl chains. The required molar ratios were obtained by mixing standard solutions of two lipids in chloroform. The chloroform was removed in vacuo and the lipid hydrated. Heating and cooling rates were approximately 10 °C/min and the heating curves shown were obtained at a ΔT scale setting of 0.1 °C/in.

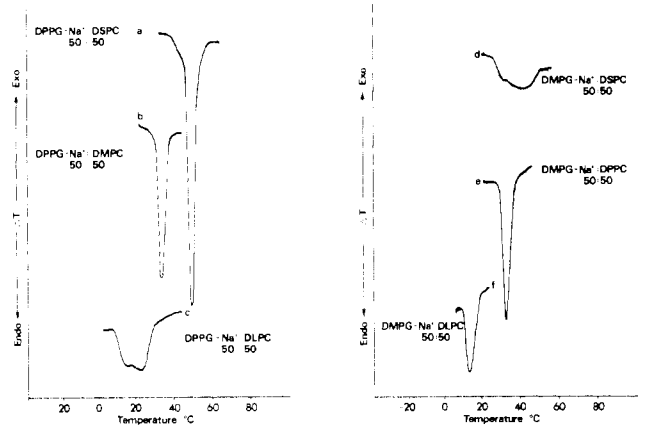

FIGURE 2: Differential thermal analysis of PG-Na^+:PC mixtures in which the two lipid species had different acyl chains. Experimental conditions were as described in Figure 1.

mixtures containing a PG-Na^+ with the corresponding PC (identical acyl chains) appeared to be completely miscible in all proportions. Each of these mixtures underwent a single narrow thermal transition at the temperature characteristic of the pure components (Figure 1). With this technique, no increase in the width of the endotherm could be detected, as compared with the width of the endotherms obtained with each of the pure components. Pretransitions similar to those observed with pure saturated PC and PG-Na^+ were observed with mixtures which contained these two lipids in various molar ratios.

DTA of Mixtures of Phosphatidylglycerol Sodium Salts and Phosphatidylcholines with Nonidentical Acyl Chains. Mixtures of PG-Na^+ with PC, in which the chain lengths of the saturated acyl chains of the two species were different by two carbon atoms, produced endotherms that were slightly broader than the endotherms observed for mixtures with identical acyl chains (Figures 2a,b,e,f). The midpoint transition temperature of a mixture of this type appears to be essentially a linear function of the transition temperatures (T_c) of the pure components and the mole fraction (X_n) such that:

$$X_1 T_{c1} + X_2 T_{c2} = T_{c\,\text{mixture}}$$

Mixtures of PG-Na^+ with PC, in which the chain lengths differed by four carbon atoms, produced endotherms with two

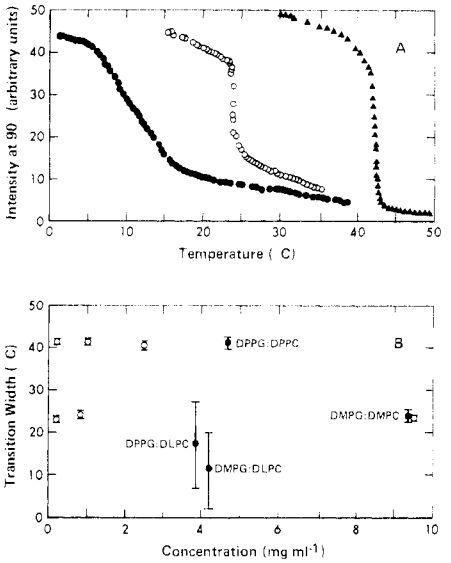

FIGURE 3: Temperature and concentration dependence of the intensity of light scattered at 90° from aqueous dispersions of phospholipids. Incident wavelength = 450 nm, heating rate 0.23 °C/min. (Panel A) Scattering curves for equimolar DMPG-Na^+:DMPC (○), DPPG-Na^+:DPPC (▲), and DMPG-Na^+:DLPC (●). (Panel B) Midpoint transition temperatures and melting ranges (transition widths) for phosphatidylcholines (○) and PG-Na^+:PC mixtures (●). The bars represent the widths from 10% to 90% of the total intensity change.

inflection points (Figures 2c,d). These inflection points were at temperatures intermediate between the transition temperatures of the pure components and varied with the composition of the particular mixture.

Temperature Dependence of the Light Scattered ($I_{90°}$) by PG-Na^+:PC Dispersions. Light scattering properties of phospholipids in aqueous dispersions were used as an alternate way of examining their phase behavior. In comparison with DTA, this technique permits a much closer approach to thermal equilibrium. In addition, it is possible to determine any concentration dependence of the transition temperature which may exist. However, no significant dependence was apparent within the concentration range studied (0.164 to 9.5 mg/mL).

Representative scans of scattered light intensity at 90° to the incident light as a function of temperature are shown in Figure 3A for DMPG-Na^+:DMPC, DPPG-Na^+:DPPC, and DMPG-Na^+:DLPC. Heating and cooling curves obtained for the same sample were superimposable. The transition widths from 10% to 90% of the intensity change were 1.7 °C (DMPG-Na^+:DMPC), 2.5 °C (DPPG-Na^+:DPPC) and 18.4 °C (DMPG-Na^+:DLPC). The corresponding values for pure DMPC and pure DPPC were 1.2 and 1.1 °C, respectively (Figure 3B). These results quantitate the observation that mixing PG-Na^+ and PC with identical chains produces only a small increase in transition width, whereas mixing nonidentical chains causes a very appreciable increase in the melting range.

DTA and DSC of Mixtures of Phosphatidylglycerol Calcium Salts and Phosphatidylcholines. The effects of Ca^{2+} on the behavior of these binary lipid systems were then investigated. A striking feature is the elimination, when mixed with 10 mol % or more PC, of the complex mesomorphism exhibited by pure PG-Ca^{2+}. Narrow thermal transitions were observed in mixtures in which the acyl chains of the PG-Ca^{2+} were the same (Figure 4b, DPPG-Ca^{2+}:DPPC) or were two carbons shorter (Figure 4a, DPPG-Ca^{2+}:DSPC) than the acyl chains of the PC, indicating the formation of a colattice of the two

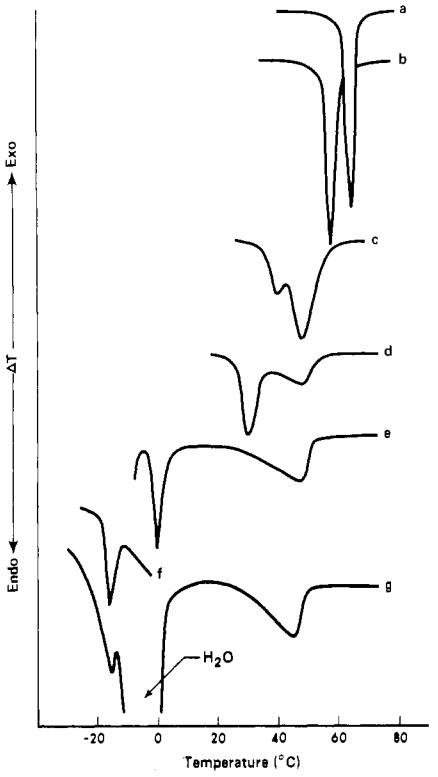

FIGURE 4: Differential thermal analysis of PG-Ca^{2+}:PC mixtures. The experimental conditions were as described for Figure 1. The lipids were hydrated with 1.0 M $CaCl_2$. (Curve a) DPPG-Ca^{2+}:DSPC; (b) DPPG-Ca^{2+}:DPPC; (c) DPPG-Ca^{2+}:DMPC; (d) DPPG-Ca^{2+}:DLPC; (e) DPPG-Ca^{2+}:DOPC; (f) DOPG-Ca^{2+}:DOPC; (g) DOPG-Ca^{2+}:DSPC. All mixtures were equimolar, except f which was 75:25 PG-Ca^{2+}:PC. In Curve f, the ice-water endotherm was removed by addition of 50% w/w ethylene glycol.

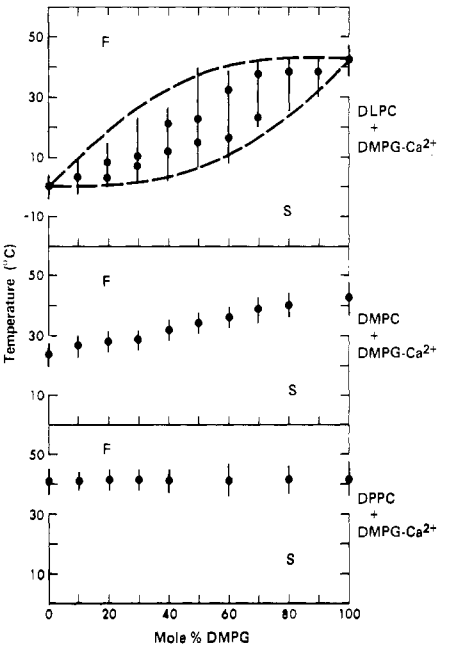

FIGURE 5: Phase diagrams for DMPG-Ca^{2+}:PC obtained by differential thermal analysis. The points (●) represent minima in the thermograms, while the bars represent total transition widths. F, fluid (liquid-crystalline) phase; S, solid (gel) phase. Dashed lines represent approximate fluidus and solidus curves. These lines have not been drawn in the two lower panels because the transition widths of the mixtures were not detectably greater than those of the pure components.

polar head groups with Ca^{2+}. The transition temperatures of the mixtures were elevated compared with the same mixtures in the absence of Ca^{2+}. Mixtures of DOPG-Ca^{2+}:DOPC exhibited a single narrow transition (Figure 4f) but the increase in transition temperature caused by Ca^{2+} was much less than that observed with saturated lipids.

When the saturated acyl chains of the PG-Ca^{2+} were longer than those of the PC, melting occurred over a much wider temperature range. Thus, the thermograms of DPPG-Ca^{2+}:DMPC and DPPG-Ca^{2+}:DLPC (Figure 4c,d) showed broad transitions with two inflection points (minima), indicative of lipid immiscibility. The mixtures DOPC:DPPG-Ca^{2+} and DOPG-Ca^{2+}:DSPC each exhibited two widely separated endotherms centered at 0 and 48 °C (Figure 4e, DOPC:DPPG-Ca^{2+}) and at −16 and 45 °C (Figure 4g, DOPG-Ca^{2+}:DSPC), respectively, consistent with even less miscibility of the components.

The behavior of mixtures of DMPG-Ca^{2+} with either DMPC, DPPC, or DLPC was investigated over a wide range of composition and phase diagrams were than constructed (Figure 4). Again, with identical chains a single narrow thermal transition was observed and a progressive elevation in the transition temperature occurred with increasing proportion of the PG-Ca^{2+} component (Figure 5, DMPG-Ca^{2+}:DMPC). In each mixture of DMPG-Ca^{2+}:DPPC a single endotherm was observed with a constant transition temperature of 41.5 °C over the whole range of composition (Figure 5, DMPG-Ca^{2+}:DPPC). It is quite evident, however, that mixtures of DMPG-Ca^{2+}:DLPC melted over a much wider range than those of DMPG-Ca^{2+}:DMPC or DMPG-Ca^{2+}:DPPC. Of the three mixtures only DMPG-Ca^{2+}:DLPC showed clear evidence of a phase separation.

The transition enthalpy for an equimolar mixture of DMPG-Ca^{2+}:DMPC was determined by DSC and found to be 7.15 kcal mol^{-1}. The enthalpy for an equimolar mixture of DMPG-Ca^{2+}:DPPC was found to be 8.83 kcal mol^{-1} (see Discussion).

Effects of Cholesterol on PG:PC Mixtures in the Presence of Ca^{2+}. Addition of a small amount of cholesterol to a DPPG-Ca^{2+}:DLPC mixture resulted in a single broad endotherm with a transition minimum at 28 °C (Figures 6b,c), between the two minima (20 and 52 °C) observed in the absence of cholesterol (Figure 6a). The phase transition of the DPPG-Ca^{2+}:DLPC mixture was completely eliminated at a phospholipid:cholesterol ratio of 2:1 (Figure 6d), in agreement with results previously reported for phosphatidylcholines (Hinz & Sturtevant, 1972) and various phospholipid mixtures (Demel et al., 1977).

Discussion

Binary mixtures of PG-Na^+ salts with PC having identical acyl chains exhibit a remarkably narrow melting range regardless of the composition of the mixtures (minimal separation of the solidus and fluidus curves of the phase diagram). The only evidence of a departure from the behavior of a pure compound is the slight increase in transition width detected by light scattering, from approximately 1 to 2 °C (Figure 3). In a two-component mixture, the solidus and fluidus curves can only merge if the two components are completely miscible and also have identical transition temperatures and enthalpies. Table I shows that these thermodynamic requirements are approached for PG-Na^+ salts and phosphatidylcholines with identical acyl chains. The same requirements are not met for mixtures of DPPC (T_c = 41.5 °C) and dipalmitoylphosphatidylserine (DPPS, T_c = 50–53 °C) and in this case a wide

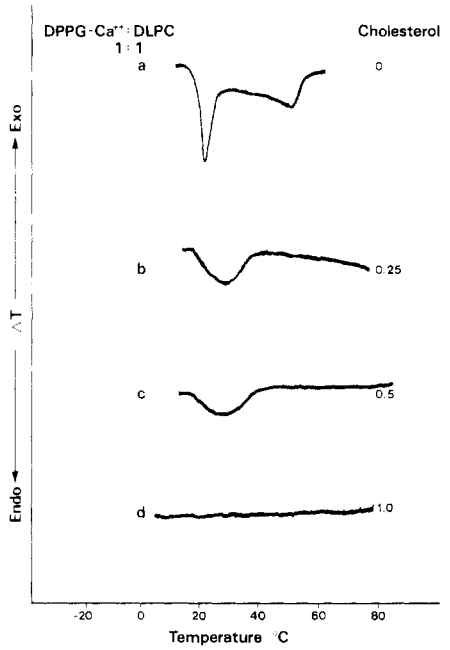

FIGURE 6: Effect of cholesterol on the thermal behavior of DPPG-Ca^{2+}:DLPC mixtures. (a) Heating curve of DPPG-Ca^{2+}:DLPC (1:1) in 1.0 M $CaCl_2$. (b) Heating curve of DPPG-Ca^{2+}:DLPC:cholesterol (1:1:0.25) in 1.0 M $CaCl_2$. (c) Heating curve of DPPG-Ca^{2+}:DLPC:cholesterol (1:1:0.5) in 1.0 M $CaCl_2$. (d) Heating curve of DPPG-Ca^{2+}:DLPC:cholesterol (1:1:1) in 1.0 M $CaCl_2$.

separation of the solidus and fluidus curves has been observed (Luna & McConnell, 1977). A further difference is the persistence of the pretransition endotherm throughout the complete range of PG-Na^+:PC mixtures. Freeze fracture electron micrographs of an equimolar mixture of DMPG-Na^+:DMPC quenched from 18 °C showed that all the visible fracture faces had the banded structure characteristic of the $P_{\beta'}$ phase (R. D. Bradley, D. G. Scraba, & P. G. Barton, unpublished work). Apparently, this structure is absent from the phase diagram of pure DPPS (Luna & McConnell, 1977).

In mixtures containing PG-Na^+ and PC with the chains of the two lipids differing by two carbon atoms, some broadening of the endotherm was noted (Figure 2). With the light scattering technique, the thermal behavior of DMPG-Na^+:DLPC in 0.1 M NaCl was manifested as a gradual change in intensity ($I_{90°}$) over a range of about 20 °C. With a difference of four carbon atoms in chain length, two minima could be detected in the thermograms, indicative of a phase separation. These results are reminiscent of studies by Chapman and co-workers (Phillips et al., 1970) relating to the mixing behavior of different synthetic phosphatidylcholines. This comparison suggests that the phase separations are primarily due to immiscibility of the hydrocarbon chain structures of the two components.

The ability of Ca^{2+} ions to induce isothermal phase separations in mixed lipid bilayers has important implications for membrane organization and function. Most investigators have therefore emphasized the ability of Ca^{2+} to increase the segregation of acidic and zwitterionic lipids (for review, see Lee, 1977). Here we report examples in which such a Ca^{2+}-induced phase separation could not be detected, namely, in mixtures of DMPG:DMPC, DMPG:DPPC, DPPG:DPPC, and DPPG:DSPC. Again, the criteria for minimizing the area between the solidus and fluidus curves would be complete miscibility of the Ca^{2+}-phosphoglycerol and phosphocholine head groups plus identical transition temperatures and enthalpies of the PG-Ca^{2+} and PC components. Unfortunately, we cannot accurately determine T_c and ΔH for DMPG-Ca^{2+} or DPPG-Ca^{2+} because of the complex mesomorphism of the pure PG-Ca^{2+}, previously mentioned. Instead, we have determined the values for equimolar mixtures of DMPG-Ca^{2+}:DMPC (T_c = 34 °C, ΔH = 7.15 kcal/mol) and DMPG-Ca^{2+}:DPPC (T_c = 41 °C, ΔH = 8.83 kcal/mol) and compared them with literature values for pure DMPC and DPPC (see Table I). It is evident from these comparisons that the mixture DMPG-Ca^{2+}:DPPC should exhibit the closest approach of the fluidus and solidus curves in the phase diagram whereas for DMPG-Ca^{2+}:DMPC the area enclosed by these curves should be appreciable. As yet, however, the expected difference could not be detected in the experimental measurement of the transition widths obtained by DTA.

In other cases (Figure 5, DMPG-Ca^{2+}:DLPC, and Figure 4c,d,e,g) phase separations were clearly evident in the thermograms. It should be noted that in each case where a phase separation was observed the temperatures of the two minima did not correspond to the transition temperatures of the two pure lipid components. This implies that each of the coexisting phases is enriched in one or other of the lipid components but to an extent less than 100%. The observed transition temperatures then presumably reflect the mole fraction of each lipid in each of the two phases, and this provides a semiquantitative assessment of the mutual miscibilities of the two lipids. In the case of DPPG-Ca^{2+}:DOPC (Figure 4g), for example, the minima are displaced only a few degrees from the T_c values of the pure components, indicating a very limited mutual miscibility.

Demel et al. (1977) have reported that, in immiscible mixtures of acidic phospholipids and phosphatidylcholines (DMPG-Na^+:DOPC (50:50); DMPG-Na^+:DOPE (50:50)), cholesterol preferentially eliminated the transition endotherm of the lower melting component. Our results with DPPG-Ca^{2+}:DLPC:cholesterol and DMPG-Ca^{2+}:DLPC:cholesterol mixtures indicated that this preferential interaction did not occur when partially miscible mixtures of phospholipids were used. Instead, addition of a small amount of cholesterol to a DPPG-Ca^{2+}:DLPC mixture apparently increased the miscibility of the PG-enriched and PC enriched components.

We conclude from our results that the polar head groups of PG and PC are highly miscible, either in the presence or absence of Ca^{2+} ions, and that the characteristic phase behavior of such mixtures is therefore determined primarily by the structures of the constituent hydrocarbon chain structures. This conclusion cannot necessarily be extended to other mixtures of acidic and zwitterionic lipids in which the polar head groups may play a more important role in inducing phase separations.

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References

Barton, P. G., & Gunstone, F. D. (1975) *J. Biol. Chem. 250,* 4470-4476.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.

Brockerhoff, H. (1965) *Arch. Biochem. Biophys. 110,* 586-592.

Chapman, D., Williams, R. M., & Ladbrooke, B. D. (1967) *Chem. Phys. Lipids 1,* 445-475.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488,* 36–42.
Dawson, R. M. C. (1967) *Biochem. J. 102,* 205–210.
Demel, R. A., Jansen, J. W. C. M., Van Dijck, P. W. M., & Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465,* 1–10.
Hinz, H-J., & Sturtevant, J. M. (1972) *J. Biol. Chem. 247,* 3697–3700.
Jackson, C. M., Owen, W. G., Gitel, S. N., & Esmon, C. T. (1974) *Thromb. Diath. Haemorrh., Suppl. 57,* 273–293.
Jacobson, K., & Papahadjopoulos, D. (1975) *Biochemistry 14,* 152–161.
King, E. J. (1932) *Biochem. J. 26,* 292–299.
Lee, A. G. (1977) *Biochim. Biophys. Acta 472,* 285–344.
Luna, E. J., & McConnell, H. M. (1977) *Biochim. Biophys. Acta 470,* 303–316.
Papahadjopoulos, D., Jacobson, K., Nir, S., & Isac, T. (1973) *Biochim. Biophys. Acta 311,* 330–348.
Papahadjopoulos, D., Vail, W. J., Pangborn, W. A., & Poste, G. (1976) *Biochim. Biophys. Acta 448,* 265–283.
Phillips, M. C., Ladbrooke, B. D., & Chapman, D. (1970) *Biochim. Biophys. Acta 196,* 35–44.
Tocanne, J. F., Ververgaert, P. H. J. Th., Verkleij, A. J., & Van Deenen, L. L. M. (1974) *Chem. Phys. Lipids 12,* 201–219.
Van Dijck, P. W. M., Ververgaert, P. H. J. Th., Verkleij, A. J., Van Deenen, L. L. M., & de Gier, J. (1975) *Biochim. Biophys. Acta 406,* 465–478.
Verkleij, A. J., de Kruyff, B., Ververgaert, P. H. J. Th., Tocanne, J. F., & Van Deenen, L. L. L. (1974) *Biochim. Biophys. Acta 339,* 432–437.
Ververgaert, P. H. J. Th., de Kruyff, B., Verkleij, A. J., Tocanne, J. F., & Van Deenen, L. L. L. (1975) *Chem. Phys. Lipids 14,* 97–101.

Ligand-Induced Conformation Changes in *Torpedo californica* Membrane-Bound Acetylcholine Receptor†

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ABSTRACT: A time-dependent increase in ligand affinity has been studied in cholinergic ligand binding to *Torpedo californica* acetylcholine receptor by inhibition of the kinetics of [^{125}I]-α-bungarotoxin–receptor complex formation. The conversion of the acetylcholine receptor from low to high affinity form was induced by both agonists and antagonists of acetylcholine and was reversible upon removal of the ligand. The slow ligand induced affinity change in vitro resembled electrophysiological desensitization observed at the neuromuscular junction and described by a two-state model (Katz, B., & Thesleff, S. (1957) *J. Physiol. 138,* 63). A quantitative treatment of the rate and equilibrium constants determined for binding of the agonist carbamoylcholine to membrane bound acetylcholine receptor indicated that the two-state model is not compatible with the in vitro results.

The binding of AcCh[1] to the nicotinic AcChR results in an increase in the cation permeability of the postsynaptic membrane, thereby initiating postsynaptic depolarization at the neuromuscular junction. The preparation of membrane fragments enriched in AcChR from *Torpedo californica* (Duguid & Raftery, 1973) allows in vitro study of the interaction of this molecule in its membrane environment with ligands that depolarize (agonists) or that prevent depolarization (antagonists) in vivo. Inhibition of the time course of AcChR-[^{125}I]-α-Butx complex formation by ligands is a convenient method for studying these in vitro interactions since receptor-α-Butx complex formation is irreversible and a direct and simple assay procedure has been developed (Schmidt & Raftery, 1973).

It has been previously shown (Katz & Thesleff, 1957) that prolonged application of the agonists AcCh, Carb, or succinylcholine to frog sartorius muscle end plates resulted in decreased effectiveness of these agonists, although they were applied in doses large enough to usually cause depolarization. They termed this phenomenon "desensitization" and explained it by showing that the AcChR changed from an "effective" (low ligand affinity) to a "refractory" (high ligand affinity) state in the presence of ligand. Rang & Ritter (1969, 1970a,b) found that preincubation with agonists caused an increase in AcChR affinity for certain antagonists which was independent of the agonists that induced this "metaphilic effect". Using AcChR-rich membranes from *Torpedo marmorata,* Weber et al. (1975) have reported that preincubation with the agonists AcCh, Carb, and PTA induced a high affinity form of AcChR, while the antagonists d-TC and flaxedil did not, with Hexa and Deca causing this change in affinity toward Carb but not toward themselves. They concluded that the transition to the high affinity form of AcChR is induced primarily by agonists.

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[1] Abbreviations used: AcCh, acetylcholine; AcChR, acetylcholine receptor; AcChE, acetylcholinesterase; α-Butx, α-bungarotoxin; Carb, carbamoylcholine; DAP, bis(3-aminopyridinium)-1,10-decane diiodide; DFP, diisopropyl fluorophosphate; Deca, decamethonium; Hexa, hexamethonium; d-TC, d-tubocurarine; PTA, phenyltrimethylammonium; *Torpedo* Ringers: 5 mM Tris-HCl buffer (pH 7.4) containing 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$; cpm, counts per minutes; [ ]$_0$ denotes total concentration.

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