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Polymorphic Phase Behavior of Cardiolipin Derivatives Studied by ^{31}P NMR and X-ray Diffraction

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ABSTRACT: The polymorphic phase behavior of cardiolipin (diphosphatidylglycerol) analogues with two to five chains per phospholipid head group, namely, dilysocardiolipin, monolysocardiolipin, cardiolipin, and acylcardiolipin, respectively, has been studied by ^{31}P NMR and X-ray diffraction. Dilysocardiolipin dispersions at low salt concentration are micellar, and a transition to a lamellar phase takes place between 1 and 2 M NaCl. From light-scattering measurements, it is also found that a transition takes place from the micellar state with a midpoint at 5.2 mM $CaCl_2$, 0.95 M $HClO_4$, and 1.5 M NaCl. Monolysocardiolipin dispersions are lamellar throughout the concentration range from zero to saturated NaCl. Cardiolipin dispersions undergo a transition from a lamellar to an inverted hexagonal phase between 1 and 2 M NaCl. Acylcardiolipin dispersions are in an inverted hexagonal phase throughout the concentration range from zero to saturated NaCl. The chemical shift anisotropies of both phosphate groups in dilysocardiolipin and of one of the phosphate groups in monolysocardiolipin are drastically reduced in the lamellar phase, indicating a different conformation of the phosphatidyl head group from that normally found in diacyl phospholipid bilayers. The results provide strong support for the "shape" concept of lipid polymorphism when viewed in its most general form including configurational entropy, hydrophobic effects, etc. and indicate the importance of head-group interactions in determining the lipid phase behavior.

Cardiolipin (diphosphatidylglycerol) is an anionic tetraacyl phospholipid found almost exclusively either in the inner mitochondrial membrane or in the plasma membrane of microorganisms. Two phosphatidyl groups are esterified to a single glycerol in the cardiolipin molecule, yielding a structure with a relatively small head group in comparison to the total hydrocarbon chain volume. In addition, the chains are frequently highly unsaturated. For example, 80–90% of the fatty acyl chains of beef heart mitochondrial cardiolipin are linoleoyl, and for yeast mitochondrial cardiolipin, the acyl chains are mostly palmitoleoyl and oleoyl.

At low salt concentration and neutral pH, beef heart cardiolipin disperses to form bilayer vesicles analogous to the lamellar lipid arrangement thought to predominate in biological membranes. It has been demonstrated, however, that an inverted hexagonal structure can be readily induced by addition of calcium or other divalent ions (Rand & Sengupta, 1972), by interaction with cytochrome *c* (de Kruijff & Cullis, 1980), and by pH titration or increasing the monovalent ion concentration (Seddon et al., 1984). This potential to form an inverted hexagonal (H_{II}) phase may have functional significance and undoubtedly arises from the unique structure of the cardiolipin molecule, in particular its large chain to head-group volume ratio. As discussed in the above references [see also Cullis et al. (1978) and Seddon et al. (1983)], repulsive interactions between the head groups, and in particular increasing hydration, tend to stabilize the lamellar phase relative to the inverted hexagonal phase. Although several other factors affect the phase behavior, it appears that when the head-group repulsions and/or head-group hydration are reduced by any of the means mentioned above, the large chain to head-group volume ratio then favors a transition to the inverted hexagonal phase. A further consequence of these assumptions is that the phase stability should also be affected by changes in the hydrophobic/hydrophilic balance and in the chain volume and chain entropy of the cardiolipin molecule.

In the present work, we have investigated the effects of modification of the cardiolipin molecular structure on the phase preference of the lipid–water dispersions, with particular reference to the salt dependence. Cardiolipin (CL)[1] analogues with different numbers of acyl chains have been studied ranging from dilysocardiolipin with two chains per head group, via monolysocardiolipin with three chains and cardiolipin itself with four chains, to acylcardiolipin with five chains. It is found that monolyso-CL forms exclusively lamellar structures and acyl-CL solely inverted hexagonal structures throughout the available range of NaCl concentrations. As previously demonstrated (Seddon et al., 1984), CL undergoes a transition from a lamellar to an inverted hexagonal phase at 1–2 M NaCl, and dilyso-CL exhibits an extremely interesting phase behavior, transforming from a micellar to a lamellar structure at approximately the same salt concentration. The dependence of this latter transition on calcium concentration and on acid concentration has also been investigated. These measurements constitute a direct test of the geometric concepts used to interpret lipid polymorphic phase behavior and demonstrate further the importance of head-group energetics in determining the phase preference.

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[1] Abbreviations: dilyso-CL, 1,3-bis[1-acyl-2-lyso-*sn*-glycero(3)-phospho]-*sn*-glycerol; monolyso-CL, 1-[1,2-diacyl-*sn*-glycero(3)-phospho]-3-[1-acyl-2-lyso-*sn*-glycero(3)phospho]-*sn*-glycerol; CL (cardiolipin), 1,3-bis[1,2-diacyl-*sn*-glycero(3)phospho]-*sn*-glycerol; acyl-CL, 1,3-bis[1,2-diacyl-*sn*-glycero(3)phospho]-2-acyl-*sn*-glycerol; diMeCL, 1,3-bis[1,2-diacyl-*sn*-glycero(3)-(3-*O*-methyl)phospho]-*sn*-glycerol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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Materials and Methods

Beef heart cardiolipin (sodium salt) was obtained from Sigma Chemical Co. (St. Louis, MO). Monolysocardiolipin derived from beef heart cardiolipin was obtained from Avanti Biochemicals (Birmingham, Al). All dispersions, except those with $CaCl_2$, contained 1 mM EDTA and were adjusted to pH 7.4.

Acylcardiolipin was synthesized by the catalyzed acylation of beef heart CL with oleic anhydride. Beef heart CL (218 mg) in 95% EtOH was mixed with 4-(dimethylamino)pyridine (22.5 mg) and taken to dryness by rotary evaporation under a N_2 atmosphere. Residual water was removed by reevaporating the mixture from CH_2Cl_2 (distilled from P_2O_5) under vacuum. Oleoyl anhydride was prepared from 128.8 mg of oleic acid (puriss. grade; Fluka, Buchs, Switzerland) which was dried 3 times using CH_2Cl_2 and taken up in 1 mL of dry CCl_4 (distilled from P_2O_5) and 514 mg of cyclohexylcarbodiimide which had been sublimed (Selinger & Lapidot, 1966). After 30 min, the above reaction mixture was filtered to remove dicyclohexylurea, mixed with the cardiolipin, and sealed at a total volume of 1–2 mL under Ar in a screw-top culture tube. After magnetic stirring overnight at room temperature, the mixture was filtered and the CCl_4 solution washed 3 times with an equal volume of 0.1 M HCl to remove catalyst and then centrifuged 3 times from equal volumes of 0.1 M $NaHCO_3$ to remove excess unreacted oleic acid. The solvent was then removed under vacuum, and the lipid was taken up in $CHCl_3$ and loaded on a 2.5 × 10 cm column of silica gel 60 (Merck, Darmstadt, FRG; 70–230 mesh, extra pure). The product was eluted from this column with $CHCl_3$/CH_3OH/NH_4OH (100:15:1 v/v) as detected by TLC in $(CH_3)_2CO$/$CHCl_3$/CH_3OH/CH_3COOH/H_2O (40:30:10:10:5 v/v). This fraction was taken to dryness, redissolved in $CHCl_3$, and chromatographed on two 20 × 20 cm TLC plates coated with silica gel 60 F254, 2 mm thick (Merck, Darmstadt, FRG), using the solvent system $CHCl_3$/CH_3OH/NH_4OH (concentrated) (65:15:1 v/v). The major UV-quenching band which ran faster than cardiolipin, but nearly cochromatographed with oleic acid, was recovered from the plates by extraction from the silica gel with $CHCl_3$/CH_3OH (1:1 v/v) to give a yield of 192 mg of acyl-CL. Structural characterization of acyl-CL produced by this reaction scheme was provided earlier by Fowler (1983).

Dilysocardiolipin was prepared from beef heart CL by hydrolysis with pancreatic phospholipase A_2 (de Haas et al., 1968). After removal of solvent, cardiolipin (500 mg) was dispersed in 200 mL of 0.1 M sodium borate (pH 7.2) to which was added 300 mg of sodium deoxycholate. After the mixture was stirred for 1 h under N_2, 0.25 mL of 2 M calcium acetate was added, followed by 0.1 mg of pancreatic phospholipase A_2 (Boehringer-Mannheim, FRG; 600 units/mg). After 10 min, the reaction was stopped by adding 1 mL of glacial acetic acid. The mixture was extracted 4 times with diethyl ether (peroxide free) to remove fatty acid and deoxycholate and then taken to dryness by rotary evaporation. The lipid was taken up in $CHCl_3$/CH_3OH/NH_4OH (100:15:1 v/v) and loaded on a 2.8 × 30 cm column of silica gel 60 (Merck, Darmstadt, FRG; 70–230 mesh, extra pure) which had been packed in $CHCl_3$/CH_3OH/NH_4OH (65:30:3 v/v) and then equilibrated with N_2-purged $CHCl_3$/CH_3OH/NH_4OH (100:15:1 v/v). The column was sequentially eluted with the following N_2-purged $CHCl_3$/CH_3OH/NH_4OH solvent mixtures: 100:15:1 v/v to remove fatty acid, 65:30:3 v/v to elute cardiolipin (trace) and monolyso-CL (34 mg), and finally 65:35:5 v/v to elute dilyso-CL (133 mg). The purity of the products was checked by TLC in $(CH_3)_2CO$/$CHCl_3$/CH_3OH/CH_3COOH/H_2O (40:30:10:10:5 v/v). No further purification was required for the dilyso-CL. The Ca^{2+} content of the dilyso-CL preparation was determined by atomic absorption spectroscopy to be 0.03 mol/mol of phosphate. Monolyso-CL contained no detectable Ca^{2+}.

DiMeCL was prepared from bovine CL by using diazomethane (*Organic Syntheses*, 1963). Dried CL was converted to the free acid with 0.1 M HCl and treated in $CHCl_3$ solution with an excess of CH_2N_2 at 0 °C. Excess reagents were removed by vacuum, and no further purification was required. Structural characterization was provided earlier by Gwak (1983).

The fatty acid composition of bovine CL was determined by GLC to consist of 87% linoleic, 5% oleic, 2% palmitic, and 1% stearic acids with 5% unidentified. Monolyso-CL and dilyso-CL derived from bovine CL were found to have linoleic acid contents of 83% and 78%, respectively.

Proton dipolar-decoupled 109-MHz ^{31}P NMR spectra were collected with a Bruker WH-270 spectrometer operating in the Fourier transform mode. Either the direct free induction decay was collected, or a 90°–τ–180°–τ spin-echo sequence was employed, with an 11-μs 90° pulse and phase cycling. The decoupling power was approximately 20 W, and the duty cycle of the gated decoupling was 0.2%. Spin–lattice relaxation times (T_1) were determined by using a 180°–τ–90° inversion recovery pulse sequence with a waiting time of 8 s between pulse trains. Values of T_1 were obtained from linear regression analysis of the τ dependence of ln $(I_\infty - I_\tau)$ where I_τ is the amplitude of the $\sigma_\perp$ peak for delay τ and I_∞ was obtained with τ = 17 s. Six τ values were used, and the quoted errors are derived from the linear regression. To within the limits set by the signal to noise ratio, the T_1 value was homogeneous throughout the entire powder pattern. That is, the inverted magnetization decayed at approximately the same rate at the $\sigma_\parallel$ shoulder as at the $\sigma_\perp$ peak. The samples were thermostated at 20 °C.

X-ray diffraction experiments were performed by using a Guinier camera with a quartz crystal monochromator to isolate the Cu $K\alpha_1$ (λ = 0.15405 nm) radiation. Exposure times for the CEA REFLEX 15 X-ray film (Ceaverken AB, Strägnäs, Sweden) were typically between 1 and 15 h. The hydrated lipid samples were sealed between mica plates in specially built metal holders. Measurements were made at room temperature.

Light scattering at 90° was recorded at 460 nm by using a Perkin-Elmer Fluorispec spectrofluorometer, and optical turbidity was recorded at the same wavelength with a Cary spectrophotometer. The concentration of dilyso-CL used for the optical measurements was ca. 0.3 mg/mL.

Results

The structures of the various cardiolipin analogues studied are given in Figure 1. The derivatives have increasing numbers of fatty acyl chains per head group, from two for dilyso-CL to three for monolyso-CL, four for cardiolipin, and five for acyl-CL. All analogues are derived from beef heart cardiolipin for which the acyl chain composition is 87% linoleoyl (Powell et al., 1985). The acyl-CL derivative has in addition an oleoyl chain acylated on the glycerol *sn*-2 position. The proton-decoupled ^{31}P high-field NMR spectra of the various derivatives suspended in 0 and 3 M NaCl, at 20 °C, are given in Figure 2.

In the absence of salt, dilyso-CL forms a clear solution and gives rise to a narrow, isotropic ^{31}P NMR line (Figure 2a). This behavior is characteristic of a micellar solution. In the

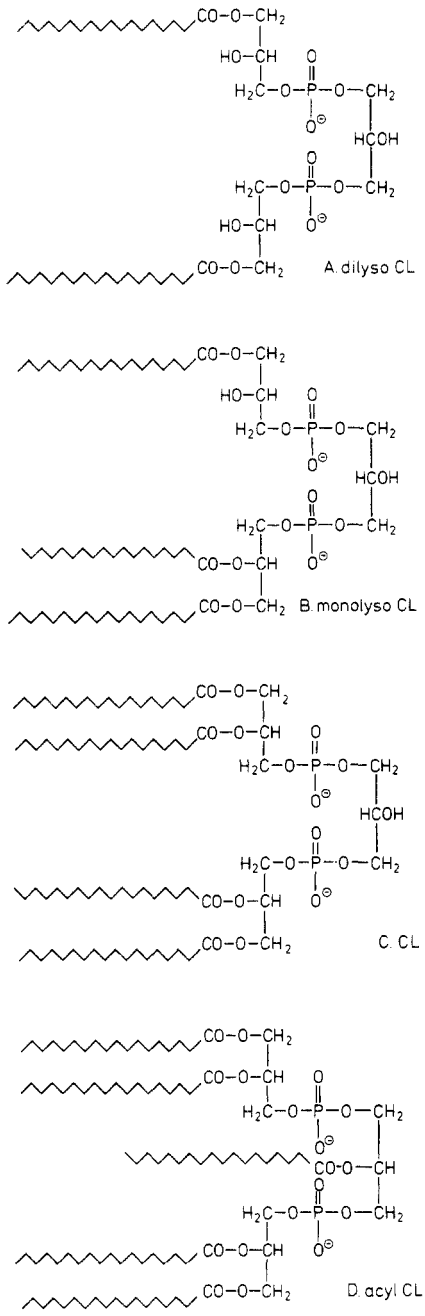

FIGURE 1: Structures of cardiolipin derivatives: (A) dilysocardiolipin; (B) monolysocardiolipin; (C) cardiolipin; (D) acylcardiolipin. The acyl chains are indicated schematically. For beef heart CL analogues, the phosphatidyl chains are 87% linoleoyl. For acyl-CL, the 2-acyl chain is oleoyl.

presence of 3 M NaCl, the dilyso-CL sample has precipitated out and in fact floats at this high salt concentration. A powder pattern with a negative chemical shift anisotropy and a shape characteristic of axially symmetric motional averaging is observed in the ^{31}P NMR spectrum (Figure 2b). This is suggestive of a lamellar lipid organization, but because of the very low chemical shift anisotropy, $\Delta\sigma \sim -8$ ppm, it is not diagnostic of bilayer formation. The chemical shift anisotropy of the salt-containing samples can be increased somewhat by repeated freezing and thawing to a value of $\Delta\sigma = -14.5$ ppm. This suggests some contribution from particle size or surface curvature to the averaging of the chemical shift anisotropy in the presence of salt. However, the limiting value of −14.5 ppm is still much smaller than the shift anisotropy of ~−40 ppm normally observed for diacyl phospholipid bilayers (Seelig,

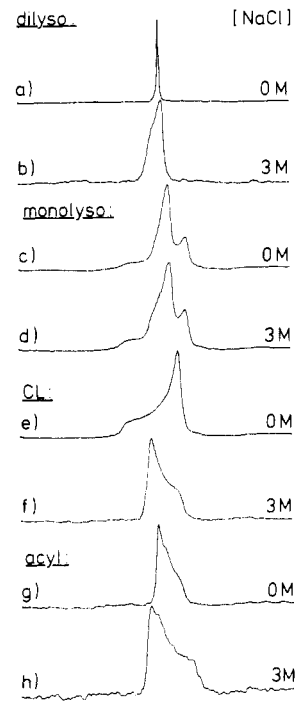

FIGURE 2: Proton dipolar-decoupled 109-MHz ^{31}P NMR spectra of cardiolipin derivatives dispersed in 0.0 and 3.0 M NaCl at 20 °C: (a) dilysocardiolipin in 0.0 M NaCl: (b) dilysocardiolipin in 3.0 M NaCl; (c) monolysocardiolipin in 0.0 M NaCl; (d) monolysocardiolipin in 3.0 M NaCl; (e) cardiolipin in 0.0 M NaCl; (f) cardiolipin in 3.0 M NaCl; (g) acylcardiolipin in 0.0 M NaCl; (h) acylcardiolipin in 3.0 M NaCl. Plot width = 16 kHz (146 ppm).

1978). The ^{31}P NMR spectrum of dry dilyso-CL at −15 °C was a characteristic nonaxial powder pattern but still showed evidence of slow motion. The maximum chemical shift anisotropy of the pattern was $|\Delta\sigma| \sim 160$ ppm, which is less than the value of ~180–190 ppm normally observed for solid phosphate diester phospholipids (Seelig, 1978). This slightly lower value is presumably due, at least in part, to residual motional averaging but nonetheless indicates that the chemical shift tensor of dilyso-CL is rather similar to those normally found and cannot account for the drastically reduced axial shift anisotropy found for dispersions at salt concentrations greater than 2 M NaCl. Monolyso-CL forms a clear gel in the absence of salt and a precipitated dispersion in the presence of salt. The ^{31}P NMR spectra are very similar in both 0 and 3 M NaCl (Figure 2c,d) and apparently consist of two superimposed axial powder patterns of chemical shift anisotropies $\Delta\sigma = -12$ and −38 ppm, respectively. The smaller shift anisotropy corresponds quite closely to that observed for dilyso-CL at the higher salt concentrations, and the larger corresponds exactly to the chemical shift anisotropy obtained from bilayers of diacyl phospholipids. Quantitation of the integrated areas under the two powder patterns in Figure 2c,d demonstrates that they correspond to approximately equal phosphate intensities. Cardiolipin itself also forms a clear gel in the absence of salt and a precipitated dispersion in the presence of salt. In the absence of salt, a typical lamellar ^{31}P NMR spectrum is obtained with a chemical shift anisotropy of $\Delta\sigma = -32$ ppm (Figure 2e). An isotropic component is sometimes observed in the CL spectrum in the absence of salt, but this can be removed by repeated freezing and thawing. In the presence of 3 M NaCl, a powder pattern characteristic of the hexagonal phase is obtained (Figure 2f) with a chemical shift anisotropy, $\Delta\sigma = +19$ ppm, which is reversed in sign and approximately half that typical of the lamellar phase. Acyl-CL does not disperse as readily in water as the other cardiolipin

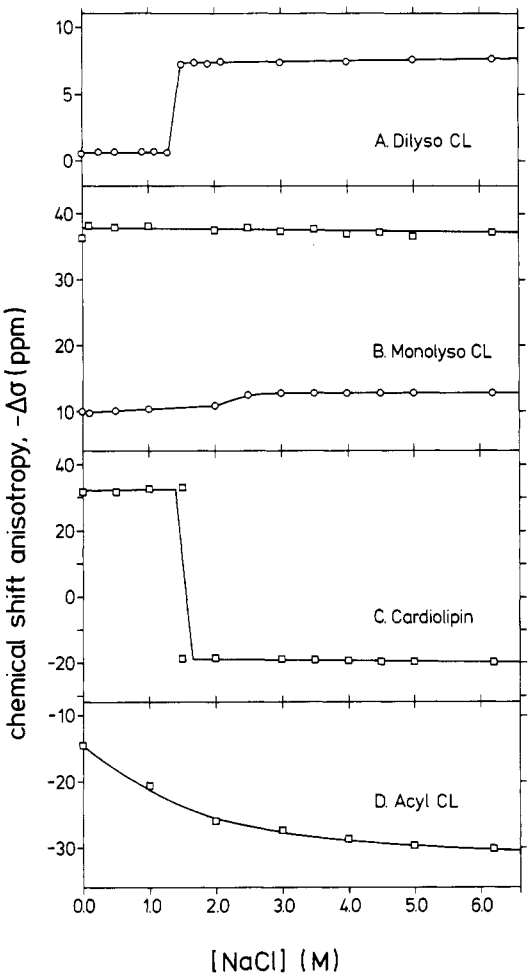

FIGURE 3: ^{31}P chemical shift anisotropy, $\Delta\sigma$, at 20 °C as a function of NaCl concentration for (A) dilysocardiolipin, (B) monolysocardiolipin, (C) cardiolipin, and (D) acylcardiolipin.

derivatives. Dispersion is facilitated by repeated freezing and thawing which removes any isotropic component in the ^{31}P spectrum. A precipitated dispersion is obtained in both the presence and absence of salt, and the ^{31}P NMR spectrum is indicative of a hexagonal phase with chemical shift anisotropies of +14.5 and +27.5 ppm in 0 and 3 M NaCl, respectively. Interestingly, diMeCL failed to disperse in water and formed an emulsion giving rise to a sharp isotropic ^{31}P NMR spectrum, characteristic of a fluid oil.

The salt dependence of the chemical shift anisotropy at 20 °C is given for all four analogues in Figure 3. For dilyso-CL, it is seen that the chemical shift anisotropy changes from zero to −14.5 ppm between 1.0 and 2.0 M NaCl, corresponding to the transition from the micellar to the presumed lamellar phase. The conversion is gradual with coexistence of both the isotropic and axial spectra over the transition range. Metastability may also be involved since the position at which the transition takes place, within the range 1.0–2.0 M NaCl, depends somewhat on the sample preparation conditions. The data given in Figure 3A apply to samples which were all frozen and thawed 3 times. For monolyso-CL, the chemical shift anisotropies of the two components change relatively little with salt concentration, as shown in Figure 3B. Presumably, monolyso-CL exists in a lamellar phase over the whole range of salt concentrations up to saturated NaCl. For cardiolipin itself, there is an abrupt change in chemical shift anisotropy between 1.0 and 2.0 M NaCl, corresponding to the transition from the lamellar to the hexagonal phase. This phase change again takes place gradually, with coexisting lamellar and

Table I: ^{31}P NMR Spin–Lattice Relaxation Times (T_1) at 109 MHz and Chemical Shift Anisotropies ($\Delta\sigma$) of Cardiolipin Derivatives at Different Salt Concentrations[a]

lipid	[NaCl] (M)	$\Delta\sigma$ (ppm)	T_1 (s)
dilyso-CL	1.4	$\lesssim$1.8[b]	0.741 ± 0.008
	1.9	$\lesssim$2.2[b]	0.701 ± 0.008
	6.2	−15.1	1.086 ± 0.015
monolyso-CL	0.0	−11.3[c]	1.287 ± 0.155
		−38.0[c]	1.258 ± 0.140
CL	0.5	−31.7	1.208 ± 0.016
	6.2	+20.1	1.294 ± 0.011

[a] T = 20 °C. [b] Line width of isotropic peak. [c] Two-component powder pattern.

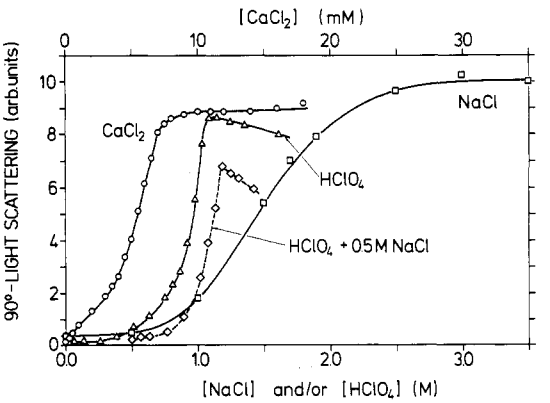

FIGURE 4: 90° light scattering at 460 nm of dilysocardiolipin dispersions (1 mg/3 mL) as a function of salt or acid concentration: (□) NaCl; (○) $CaCl_2$ [note 100-fold expansion of (upper) concentration scale]; (△) $HClO_4$; (◇) $HClO_4$ in the presence of 0.5 M NaCl [note that the horizontal scale is the total concentration ([$HClO_4$] + [NaCl]) in this case]. Dispersions contained 1 mM EDTA (except for $CaCl_2$), pH 7.4.

hexagonal ^{31}P NMR spectra throughout the transition region. After the transition, the chemical shift anisotropy remains relatively constant with increasing salt concentration. For acyl-CL, the chemical shift anisotropy is positive, corresponding to a hexagonal phase, throughout the range of salt concentrations. The absolute value of the anisotropy does increase, however, between 0 and 2.0 M NaCl, corresponding to the range in which phase transitions are observed for dilyso-CL and CL. Above 2.0 M NaCl, the chemical shift anisotropy then increases relatively slowly with increasing salt concentration.

The ^{31}P spin–lattice relaxation times at 20 °C have been measured for the various analogues in the various phases. The results are given in Table I. Monolyso-CL and cardiolipin have rather similar values of T_1, although the errors for the former determination are rather large. To within experimental error, there is no difference in T_1 between the two components of the monolyso-CL spectrum. For cardiolipin, there is a small but significant increase in T_1 on going to the hexagonal phase at high salt concentration. The T_1 values for dilyso-CL are all smaller than those for the two other derivatives. In the micellar state, the T_1 of dilyso-CL is yet shorter than in the presumed lamellar state at high salt concentration. It should, however, be mentioned that at 1.4 and 1.9 M NaCl there was a (small) coexisting lamellar component in the spectrum of dilyso-CL.

The micellar to (presumed) lamellar transition with dilyso-CL has been further studied using light scattering. The 90° light scattering at 460 nm as a function of salt or acid concentration is given in Figure 4. A broad increase in scattered light intensity is observed between 1.0 and 2.0 M NaCl, in agreement with the ^{31}P NMR results. Below this

Table II: X-ray Diffraction Repeat Spacings from Aqueous Dispersions of Cardiolipin Derivatives as a Function of Salt Concentration at Room Temperature

lipid	[NaCl] (M)	$d(hkl)$ (nm) (100)	(110)	(200)	(300)	(400)	(500)[c]	phase[a]
dilyso-CL	0.5	–	–	–	–	–	–	M
	3.0	4.5	–	–	1.5	–	–	L
monolyso-CL	3.0	4.7	–	–	1.6	1.2	1.0	L
CL[b]	0.5	6.25						L
	2.0	4.9						L
	4.0	6.1	3.5					H
acyl-CL	0.5	5.3	3.0	2.6	–	–	–	H
	3.0	(4.4)	–	–	–	–	–	–

[a] Abbreviations: M, micellar; L, lamellar; H, hexagonal. [b] Data from Seddon et al. (1983); several orders were observed, but only the fundamental (first-order) spacing is included for comparison purposes. [c] Integral multiples of Miller indices (hkl) are to be understood as higher order reflections.

range, a transparent micellar solution is obtained, and there is essentially no scattered light intensity. A similar result is obtained with $CaCl_2$, but the increase in scattering occurs at much lower concentrations. The midpoint of the NaCl-induced change is at 1.5 M, whereas that for $CaCl_2$ is at 5.2 mM. The acid $HClO_4$ is less effective than $CaCl_2$ but more effective than NaCl in inducing the transition, giving a midpoint at 0.94 M. Adding 0.5 M NaCl gives an intermediate result with a midpoint at 1.05 M acid plus salt, i.e., at 0.55 M $HClO_4$.

The identification of the lipid–water phases by ^{31}P NMR has been confirmed by X-ray diffraction. The repeat distances deduced from the low-angle diffractions of the various cardiolipin analogues in different salt concentrations are given in Table II. For a lamellar ordering, the separation between lattice planes is given by

$$d(hkl) = a/h \tag{1}$$

and that for hexagonal ordering by

$$d(hkl) = \frac{\sqrt{3}}{2} \frac{a}{\sqrt{h^2 + hk + k^2}} \tag{2}$$

where a is the lattice constant and (hkl) are the Miller indices. The indexing of the reflections is made according to either one of these two equations in Table II.

For dilyso-CL in 0.5 M NaCl, no reflections were observed under conditions for which reflections were clearly seen at 3.0 M NaCl. On the other hand, lamellar reflections have been observed for CL at 0.5 M NaCl (Seddon et al., 1983). This lack of diffraction from dilyso-CL at 0.5 M NaCl is consistent with the lack of long-range order in the micellar state. For dilyso-CL at 3.0 M NaCl, both first and third diffraction orders were observed, which are consistent with a lamellar arrangement of fundamental spacing of 4.5 nm. For monolyso-CL at 3.0 M NaCl, first, third, fourth, and fifth orders of a lamellar diffraction pattern were observed, with a fundamental repeat distance of 4.7 nm. The 1.0-nm reflection could in principle be consistent with either the second order of a (210) reflection or the third order of a (110) reflection of a hexagonal lattice for which the lower orders are not seen, although the fifth order of a lamellar pattern provides marginally better agreement. However, the ^{31}P NMR spectra discussed above leave little doubt that monolyso-CL has a lamellar arrangement. The close similarity with the diffraction spacings of dilyso-CL at 3.0 M NaCl, for which fewer reflections are observed, further confirms the lamellar ordering of the latter. For acyl-CL at 0.5 M NaCl, three clear diffractions were observed with spacings in the ratio $1/1$, $1/\sqrt{3}$, and $1/2$ which index on a hexagonal lattice as indicated in Table II. Taken together with the ^{31}P NMR results, this is good evidence that acyl-CL forms inverted hexagonal structures. At 0.5 M NaCl, the fundamental spacing is 5.3 nm, corresponding to a lattice constant (separation between cylinder centers) of 6.1 nm. At 3.0 M NaCl, only a very weak diffraction was obtained from acyl-CL which was consistent with a first-order spacing of 4.4 nm (hexagonal lattice constant $\sim$5.1 nm). The fundamental spacings observed previously (Seddon et al., 1983) for the lamellar diffractions of CL at 0.5 and 2.0 M NaCl and for the hexagonal diffractions of CL at 4.0 M NaCl are also included for comparison purposes in Table II.

Discussion

The cardiolipin analogues studied display a rich polymorphic phase behavior varying from normal micelles to inverted hexagonal phases. The ability of ions, particularly divalent ions, to trigger these phase transitions is also of considerable interest. From a spectroscopic viewpoint, the different analogues also exhibit an interesting pattern of chemical shift anisotropies, corresponding to different motions and/or different head-group structures. The putative phase assignments made on the basis of the ^{31}P NMR spectra have been confirmed by X-ray diffraction analysis which allows interpretation of the further reductions in chemical shift anisotropy in terms of motional properties of the phospholipid head-group regions.

^{31}P NMR, light scattering, and X-ray diffraction all confirm the micellar structure of dilyso-CL dispersions at salt concentrations up to 1.0 M NaCl. Between 1.0 and 2.0 M NaCl, a transition takes place to the lamellar state. In the lamellar state at 3.0 M NaCl, the bilayer repeat spacing of 4.5 nm is slightly smaller than that for monolyso-CL, which in turn is slightly smaller than that for CL itself. These small decreases are just outside the experimental error and presumably arise from changes in chain packing resulting from the reduced hydrocarbon chain volume per head group and possibly also from somewhat different degrees of hydration.

The reduced ^{31}P NMR chemical shift anisotropy of dilyso-CL in the lamellar state, relative to the usual value of $\Delta\sigma \sim -40$ to -50 ppm found for bilayer-forming lipids, is not due to an intrinsically smaller chemical shift anisotropy of the phosphate group of the lyso compound. This is seen from measurements on the dry lipid (albeit still in a somewhat fluid phase) which indicate that the chemical shift tensor is not greatly different, if at all, from that of normal diacyl phospholipids. A similar conclusion has been reached from a comparison of the spectra of dry powders of lysophosphatidylcholine and of phosphatidylcholine (van Echteld et al., 1981). The reduced anisotropy of the dilyso-CL spectrum most probably arises from a different orientation of the phosphate head group and/or glycerol backbone. Evidence

for the latter comes from comparison with monolyso-CL for which one spectral component has a small chemical shift anisotropy similar to that found for dilyso-CL, whereas the other component has the more normal value of $\Delta\sigma \sim -38$ ppm. The fact that both spectral components for monolyso-CL have approximately the same intensity strongly suggests that the two components correspond to the two inequivalent phosphate groups in the monolyso-CL molecule. To within experimental error, both components have the same spin–lattice relaxation time which unfortunately does not aid in further identification. Since both phosphate groups are part of the same molecule, the inequivalent chemical shift anisotropies suggest that the conformation of the monoacylphosphatidyl group is different from that of the diacyl group and from that found normally in bilayers of diacyl phospholipids. In particular, it should be noted that the reduced anisotropy of the second component cannot be accounted for by the effects of surface curvature or small particles, since the other component displays the full lamellar value. The conformation of the two phosphatidyl groups in dilyso-CL is presumably also similar to that of the monoacyl group in monolyso-CL, giving rise to increased motional averaging of the ^{31}P chemical shift anisotropy. It is worthwhile to note that a reduced chemical shift anisotropy of $\Delta\sigma \sim -20$ to -25 ppm has also been observed for lysophosphatidylcholine in the gel phase or in a 1:1 mixture with cholesterol (van Echteld et al., 1981). This reduction in chemical shift anisotropy was interpreted as most probably being due to a difference in conformation of the glycerol backbone of the phosphatidyl moiety.

The ^{31}P NMR spectra of cardiolipin itself indicate a transition from a conventional lamellar pattern with $\Delta\sigma \sim -32$ ppm to a conventional hexagonal pattern with $\Delta\sigma \sim +19$ ppm between 1 and 2 M NaCl. This is similar to the effects of Ca^{2+} on the NMR spectra observed previously (de Kruijff & Cullis, 1980) and confirms the previous X-ray demonstration of the salt-induced lamellar–hexagonal transition (Seddon et al., 1983). The ^{31}P NMR spectra of acyl-CL are similar to those observed for CL in high salt and correspond to those routinely found for inverted hexagonal phases. Interestingly, the chemical shift anisotropy increased with increasing salt concentration, corresponding to a decreased degree of motion or a change in the orientation of the phosphate group. The X-ray diffraction pattern from acyl-CL in 0.5 M NaCl was diagnostic of a hexagonal phase, confirming the assignment by ^{31}P NMR. The fundamental repeat spacing was 5.3 nm, of the same order as those for the lamellar phases but considerably smaller than the value of 6.9 nm found for CL at 2 M NaCl, the lowest salt concentration for which the hexagonal phase was observed (Seddon et al., 1983). This decreased spacing most probably results from a decreased degree of hydration corresponding to the difficulty found in dispersing acyl-CL and presumably arises from the increased degree of hydrophobicity of this analogue. A smaller fundamental spacing of 4.4 nm was found for acyl-CL at 3 M NaCl, as expected from the increased salt concentration [cf. Seddon et al. (1983)].

The occurrence of the micellar state for dilyso-CL adds another dimension to the polymorphism of cardiolipins. Figure 4 indicates that the transition from the micellar phase can be triggered with differing efficiencies by a variety of cations. The cation selectivity is complex, presumably involving a combination of ion binding and ionic screening. At the midpoint of the $CaCl_2$-induced transition, the ionic strength is only 0.016, and thus the ionic screening is far from complete. The Ca^{2+} ions clearly bind to the phospholipid phosphate groups, giving rise to charge neutralization with an effective binding constant $K_{app} \sim 5$ mM. This corresponds to an intrinsic binding constant, allowing for electrostatic surface enhancements, of $K_0 \sim 3$ M. For the NaCl-induced transition, the midpoint occurs at an ionic strength of ca. 1.5, at which point the ionic screening should be almost complete, but there should be relatively little binding (cf. the intrinsic binding constant for Ca^{2+}). For the acid-induced transition, the midpoint occurs at 0.94 M $HClO_4$, which is surprising since the phosphate group might be expected to have a $pK_a \sim 2.9$ (Watts et al., 1978). The lamellar–hexagonal transition in CL has previously been found to occur at $pK_a \sim 2.5$ in 1 M NaCl (Seddon et al., 1983). However, titrations of dilyso-CL with HCl (as opposed to the results for $HClO_4$ in Figure 4) have detected no change in optical clarity down to pH $\sim$0. It would thus appear that ionic screening plays a large part in the acid-induced transition in Figure 4, since the midpoint occurs at an ionic strength of $\sim$0.94. This is supported by the synergistic effect of added 0.5 M NaCl which shifts the midpoint of the transition to 0.55 M $HClO_4$, rather than increasing the midpoint concentration of the acid-induced transition as would be the case for a salt-induced screening of the acid pK_a. NaCl is, however, less effective than $HClO_4$ as seen from comparison of the curves for $HClO_4$ and NaCl alone, and this gives rise to an increased midpoint of 1.05 M for the total ionic concentration of the mixture, [$HClO_4$] + [NaCl], compared to $HClO_4$ alone.

A point of general interest concerns the temperature sensitivity of the phases, namely, how close the lipids are to a thermal phase transition. In low salt, cardiolipin itself remains in the lamellar phase up to temperatures of at least 50 °C (J. M. Seddon, personal communication). Similarly, the ^{31}P NMR spectrum of dilyso-CL in saturated NaCl does not change appreciably at temperatures up to 70 °C. Acyl-CL in the absence of salt retains the typical inverted hexagonal ^{31}P NMR pattern down to 0 °C. Thus, it ms likely that the lipids are not particularly close to a thermal phase transition, which could be sensitively triggered by added salt, for example. This view is also supported by the fact that modest salt concentrations (<1 M NaCl), which will screen a major proportion of the head-group electrostatic repulsion (Seddon et al., 1983; Cevc et al., 1980), do not induce phase changes at 20 °C. Only when the salt concentration is increased to levels at which an effective dehydration of the head groups probably also begins to take place [cf. Cevc et al. (1981) and Seddon et al. (1983)] do the phase transitions take place.

In summary, the present data on the phase behavior of lipid molecules with systematically varied structure provide strong support for the "shape" concept of lipid polymorphism[2] (Cullis & de Kruijff, 1979; Israelachvili et al., 1980). A progressive increase in the number of hydrocarbon chains in the cardiolipin analogues gives rise to a change in the equilibrium lipid–water structure which covers the entire range of lyotropic lipid polymorphism from normal micelles to inverted hexagonal phases. The ionic effects on the phase behavior illustrate further the importance of head-group interactions, in addition to simple shape considerations, in determining the lipid phase behavior and possibly also biomembrane structure.

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[2] "Shape" is taken in this context to mean the dynamic (or even thermodynamic) shape, allowing for such factors as configurational entropy and the balance of intermolecular forces acting on the various parts of the molecule.

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REFERENCES

Cevc, G., Watts, A., & Marsh, D. (1980) *FEBS Lett. 120*, 267–270.

Cevc, G., Watts, A., & Marsh, D. (1981) *Biochemistry 20*, 4955–4965.

Cullis, P. R., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Cullis, P. R., Verkleij, A. J., & Ververgaert, P. H. J. Th. (1978) *Biochim. Biophys. Acta 513*, 11–20.

de Haas, G. H., Postema, N. M., Nieuwenhuizen, W., & van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta 159*, 103–117.

de Kruijff, B., & Cullis, P. R. (1980) *Biochim. Biophys. Acta 602*, 477–490.

Fowler, W. T. (1983) Ph.D. Thesis, Clemson University, Clemson, SC.

Gwak, S. H. (1983) Ph.D. Thesis, Clemson University, Clemson, SC.

Israelachvili, J. N., Marcelja, S., & Horn, R. G. (1980) *Q. Rev. Biophys. 13*, 121–200.

Organic Syntheses, Collect. Vol. IV (1963), pp 250–253, Wiley, New York.

Powell, G. L., Knowles, P. F., & Marsh, D. (1985) *Biochim. Biophys. Acta* (in press).

Rand, R. P., & Sengupta, S. (1972) *Biochim. Biophys. Acta 255*, 484–492.

Seddon, J. M., Kaye, R. D., & Marsh, D. (1983) *Biochim. Biophys. Acta 734*, 347–352.

Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.

Selinger, Z., & Lapidot, Y. (1966) *J. Lipid Res. 7*, 174–175.

van Echteld, C. J. A., de Kruijff, B., Mandersloot, J. G., & de Gier, J. (1981) *Biochim. Biophys. Acta 649*, 211–220.

Watts, A., Harlos, K., Maschke, W., & Marsh, D. (1978) *Biochim. Biophys. Acta 510*, 63–74.

Mechanism of Activation of Phenylalanine and Synthesis of P^1,P^4-Bis(5′-adenosyl) Tetraphosphate by Yeast Phenylalanyl-tRNA Synthetase†

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ABSTRACT: The activation of L-phenylalanine by yeast phenylalanyl-tRNA synthetase using adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate is shown to proceed with inversion of configuration at P_α of ATP. This observation taken together with the lack of positional isotope exchange when adenosine 5′-[β,β-$^{18}O_2$]triphosphate is incubated with the enzyme in the absence of phenylalanine and in the presence of the competitive inhibitor phenylalaninol indicates that activation of phenylalanine occurs by a direct "in-line" adenylyl-transfer reaction. In the presence of Zn^{2+}, yeast phenylalanyl-tRNA synthetase also catalyzes the phenylalanine-dependent hydrolysis of ATP to AMP and the synthesis of P^1,P^4-bis(5′-adenosyl) tetraphosphate (Ap_4A). With adenosine 5′-[(*S*)-α-^{17}O,α,α-$^{18}O_2$]triphosphate, the formation of AMP and Ap_4A is shown to occur with inversion and retention of configuration, respectively. It is concluded that phenylalanyl adenylate is an intermediate in both processes, Zn^{2+} promoting AMP formation by hydrolytic cleavage of the C–O bond and Ap_4A formation by displacement at phosphorus of phenylalanine by ATP.

Phenylalanyl-tRNA synthetase belongs to the tetrameric class of aminoacyl-tRNA synthetases and consists of non-identical subunits ($\alpha_2\beta_2$) (Söll & Schimmel, 1974; Schimmel & Söll, 1979). Like other aminoacyl-tRNA synthetases, phenylalanyl-tRNA synthetase first activates L-phenylalanine with MgATP to give enzyme-bound phenylalanyl adenylate, which then charges its cognate tRNA (Kim et al., 1977). It is not, however, necessary to have $tRNA^{Phe}$ present during the activation step:

$$E + Phe + MgATP \rightleftarrows E{\cdot}Phe{\sim}AMP + MgPP_i$$

$$E{\cdot}Phe{\sim}AMP + tRNA^{Phe} \rightleftarrows E + Phe\text{-}tRNA^{Phe} + AMP$$

It has been suggested that the mechanism of activation of amino acids by aminoacyl-tRNA synthetases should involve the initial formation of an adenylyl–enzyme intermediate (Spector, 1982). In order to investigate the mechanism of activation of L-phenylalanine by phenylalanyl-tRNA synthetase from yeast, the stereochemical course of nucleotidyl transfer has been investigated. Positional isotope exchange experiments with adenosine 5′-[β,β-$^{18}O_2$]triphosphate have also been undertaken. Together, these experiments indicate that the activation of L-phenylalanine occurs by a direct "in-line" adenylyl-transfer reaction.

Phenylalanyl-tRNA synthetase from yeast also synthesizes P^1,P^4-bis(5′-adenosyl) tetraphosphate (Ap_4A), especially in the presence of Zn^{2+} (Plateau et al., 1981; Plateau & Blanquet, 1982; Blanquet et al., 1982). Ap_4A, first reported in a biological system by Zamecnik et al. (1966) [for review, see Zamecnik (1983)], was shown to be ubiquitous in living cells (Zamecnik et al., 1967) and appears to play an important role in protein biosynthesis (Rapaport & Zamecnik, 1976). The intracellular level of Ap_4A is directly related to the proliferative activity of the cell and on addition to permeabilized baby hamster kidney cells arrested in the G_1 phase of the cell cycle results in stimulation of DNA synthesis (Grummt, 1978).

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