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PHASE TRANSITION CHARACTERISTICS OF DIPHOSPHATIDYLGLYCEROL (CARDIOLIPIN) AND STEREOISOMERIC PHOSPHATIDYLDIACYLGLYCEROL BILAYERS

MONO- AND DIVALENT METAL ION EFFECTS

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Summary

Synthesis and phase transition characteristics of aqueous dispersions of the homologous (12 : 0, 14 : 0, 16 : 0) diphosphatidylglycerols (cardiolipins) and phosphatidyldiacylglycerols are reported. Electron microscopy of the negatively stained aqueous dispersions reveals a characteristic lamellar structure suggesting that these phospholipid molecules are organized as bilayers in the aqueous dispersions. The phase transition temperature (T_m) and the enthalpy of transition (ΔH) increase monotonically with chain length in the cardiolipin and phosphatidyldiacylglycerol series; T_m for phosphatidyldiacylglycerol is higher than that for cardiolipin of the same chain-length. The transition temperatures for the enantiomeric *sn*-3,3- and *sn*-1,1-phosphatidyldiacylglycerol and for the diastereomeric, meso-*sn*-1,3-phosphatidyldiacylglycerol are approximately the same. The molar enthalpy for the transition of cardiolipin-NH_4^+ bilayers is approximately twice the value for the phosphatidylcholines of the same chain length, i.e., the molar enthalpy per acyl chain is approximately the same in the two systems. The transition temperatures for metal ion salts of C_{16}-cardiolipin exhibit a biphasic dependence upon the unhydrated ionic radii, i.e.,

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Abbreviation: CEP, cyclic enediol phosphoryl.

the highest T_m is observed for Ca^{2+}-cardiolipin and decreases for the salts of ions with smaller and larger ionic radii than that of Ca^{2+}. The lowest T_m is observed for Rb^+-cardiolipin. Monovalent metal salts of cardiolipin exhibit two phase transitions. This effect may result from different conformational packing of the four acyl chains due to differences in metal-phosphate binding.

Introduction

Diphosphatidylglycerol or cardiolipin [1] is unique in the sense that it has four acyl chains in the same molecule [2,3] (Fig. 1A). All other naturally occurring phosphoglycerides contain a maximum of two acyl chains attached to the polar headgroup [4]. Cardiolipin occurs as a major component of mitochondrial membranes [5—7]. Besides acting as a structural component in the bilayer region, cardiolipin could also regulate the activities of membrane-bound enzymes [7—11], although the functional role of this phospholipid has not been established [12]. Recently, cardiolipin has been utilized in a procedure to extract F_1-ATPase from several mitochondrial ATPase complexes that differ in magnesium content, ATPase activity, and susceptibility to inhibitor [13]. The same group of investigators [14] have stressed the role played by membrane-bound Mg^{2+} in maintaining the ATPase complex organization. These considerations suggest the possible existence of specific types of interactions between cardiolipin and the two divalent cations, Mg^{2+} and Ca^{2+}.

This paper describes the thermotropic phase transition characteristics of aqueous dispersions of homologous cardiolipin salts containing the same cation, and a number of palmitoyl cardiolipin salts derived from monovalent and divalent metal ions. In addition, we report analogous data for another type of acidic phospholipid containing four acyl chains, but one phosphodiester func-

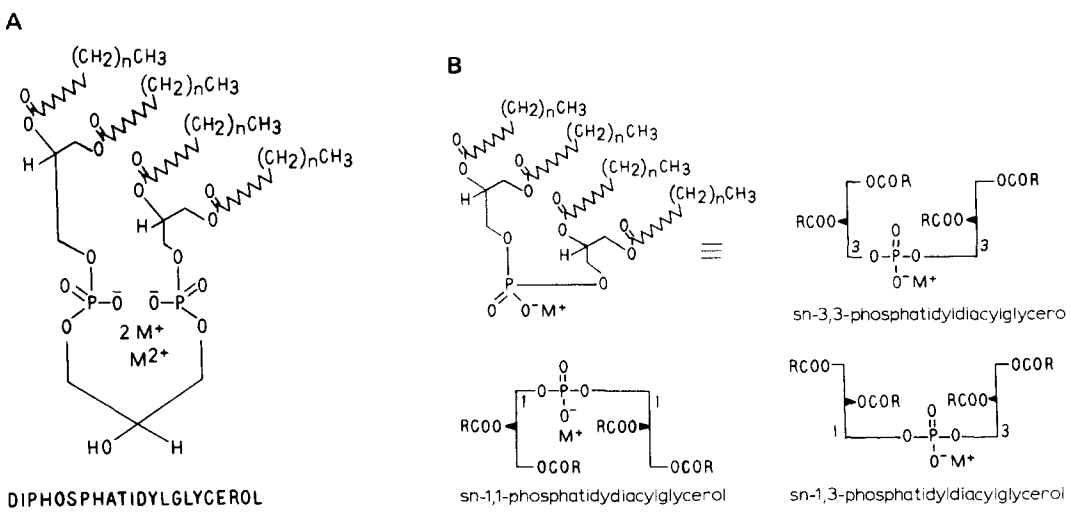


Fig. 1. (A) Diphosphatidylglycerol and (B) stereoisomeric phosphatidyldiacylglycerols. (No conformational relationship between polar head group and acyl chain is implied.)

tion, i.e., phosphatidyldiacylglycerol (Fig. 1B). We have synthesized the phosphatidyldiacylglycerol molecule as its three possible stereoisomers: the *sn*-3,3 and *sn*-1,1 enantiomers and the *sn*-1,3 diastereomer (meso-form). This permits an examination of possible effects of phospholipid configuration on phase transition characteristics.

Materials and Methods

1,2-Diacylglycerols were prepared from freshly distilled acyl chlorides (>99% purity) and the respective 3-*O*-benzyl-*sn*-glycerol and 1-*O*-benzyl-*sn*-glycerol by known procedures [15—19]. The samples were recrystallized twice under conditions which do not promote acyl migration; purity was checked by mp and TLC (silica gel Merck No. 7736 on 5 × 20 cm plates; chloroform/acetone, 24 : 1).

Ammonium salts of cardiolipin were synthesized by the cyclic enediol phosphoryl (CEP-X) method [20]. Purification was achieved by silica gel column chromatography at the phosphotriester stage, and by DEAE-cellulose chromatography at the phosphodiester stage, by techniques already described [21]. Metal ion salts of cardiolipin were prepared by treatment of the ammonium salt with an aqueous solution of the appropriate inorganic salt in a suitable mixture of chloroform, methanol and water, as described [21]. The new compounds are listed in a Table filed with the Data Bank.

P-Cardiolipin · spermine (1 : 1) and (2 : 1) were obtained as follows: A solution containing 0.151 or 0.302 mmol of *P*-cardiolipin · 2 NH_4^+ · 2 H_2O in 45 ml $CHCl_3/CH_3OH$ (2 : 1, v/v) was mixed with 20 ml $CHCl_3/CH_3OH$/2 N aq. HCl (3 : 48 : 47) at 25°C. The mixture was stirred for a few minutes and the upper layer discarded. The procedure was repeated twice more, the lower layer was washed with $CHCl_3/CH_3OH/H_2O$ (3 : 48 : 47) and immediately treated with 0.151 mmol spermine (in both cases) dissolved in $CHCl_3/CH_3OH$ (2 : 1). The solution was evaporated and the residue freeze-dried and dried in vacuum to constant weight.

The sodium salts of the stereoisomeric phosphatidyldiacylglycerol were synthesized by the CEP-Cl (2-chloro-4,5-dimethyl-2-oxo-1,3,2-dioxaphosphole) variation [22] of the CEP-method, as follows. Step 1: reaction of CEP-Cl [23] with 1,2-di-*O*-lauroyl-, 1,2-di-*O*-myristoyl-, and 1,2-di-*O*-palmitoyl-*sn*-glycerol [15,19] in anhydrous diethyl ether, in the presence of triethylamine, afforded the corresponding cyclic triester (1,2-diacylglycero-3-*O*-CEP), which was isolated but not purified. Step 2: reaction of the 1,2-diacylglycero-3-*O*-CEP with the corresponding 1,2-diacylglycerol in anhydrous dichloromethane, in the presence of triethylamine, gave the respective bis-1,2-diacylglycerol-3-oxo-2-butyl phosphate, $(RO)_2P(O)OAcn$, which was purified by silica gel column chromatography [21]. Step 3: removal of the 3-oxo-2-butyl phosphate-blocking group in pyridine/water (1 : 1, v/v) in the presence of triethylamine gave the diester $(RO)_2P(O)OEt_3NH^+$. The diester was purified by silica gel column chromatography and converted into the sodium salt by treatment with aqueous NaCl in chloroform/methanol/water as described [21]. Utilization of the enantiomeric 2,3-diacylglycerol [16—19] in step 2 of this procedure afforded the 3,1-phosphatidyldiacylglycerol · Na^+ diastereomer. Utilization of

the 2,3-diacylglycerol enantiomer in step 1 of the above sequence gave the respective 2,3-diacylglycero-1-OCEP, which upon coupling with 2,3-diacylglycerol or with 1,2-diacylglycerol in step 2, led to members of the 1,1-phosphatidyldiacylglycerol · Na^+, and 1,3-phosphatidyldiacylglycerol · Na^+ (= 3,1-phosphatidyldiacylglycerol · Na^+) families, respectively. The symmetrical 3,3- and 1,1- families were also made directly from 2 mol diacylglycerol and 1 mol CEP-Cl or of the corresponding pyrophosphate, CEPOCEP [20,21], without isolation of the diacylglycero-CEP.

The following values: Mp = 83—85° and 102—103°, $[\alpha]_D^{20}$ = +6.45° and +5.75° (c 2.00, $CHCl_3$) for M-3,3-phosphatidyldiacylglycerol · Na^+ and *P*-3,3-phosphatidyldiacylglycerol · Na^+ should replace the slightly different values originally given for these compounds in Ref. 21.

Differential scanning calorimetric studies. To a known amount (approx. 1 mg) of the lipid, 10 μl of distilled water was added, and the aluminum sample pan for differential scanning calorimetry was sealed. The pan was then heated to 345 K and allowed to equilibrate for 10—15 min in the sample compartment of the Perkin-Elmer DSC-1B. The samples were usually cooled to below 300 K and then scanned on heating and cooling cycles at 1.25 or 2.5 K/min at the sensitivity of 1. Samples with transition temperatures higher than 345 K were scanned to 370 K. The transition profiles obtained by repeated scans were indistinguishable. From such profiles we obtained T_m (temperature at the midpoint of the transition), ΔH (enthalpy of the transition), and n (the size of the cooperative unit) by the procedures described elsewhere [24].

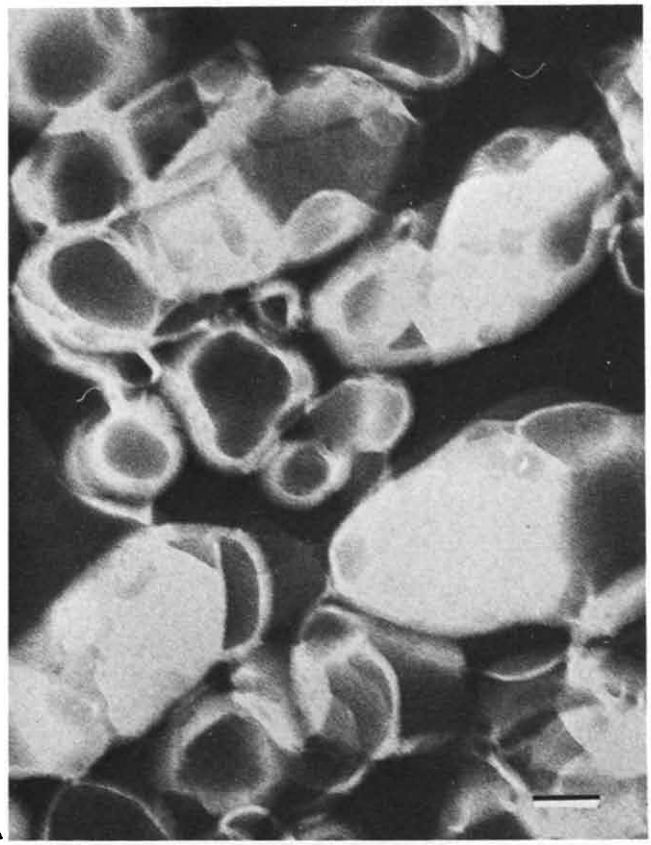
Results

Electron microscopy of phospholipid dispersions

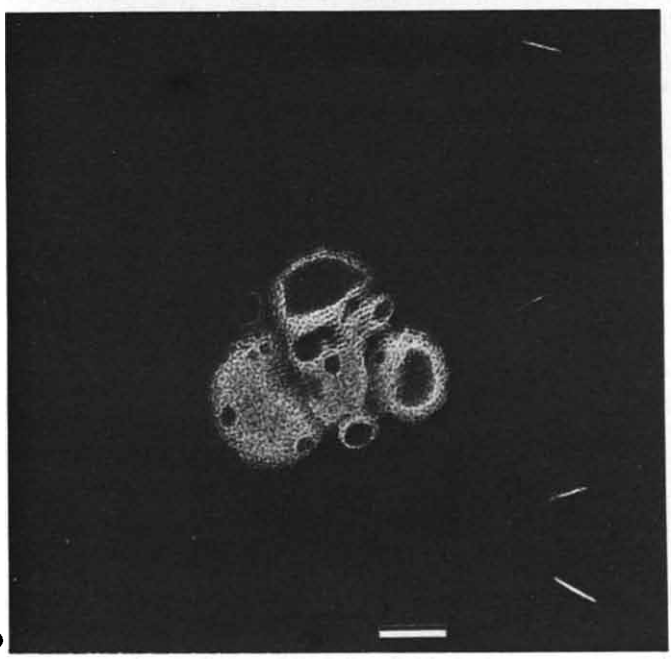
Negatively stained aqueous dispersions of palmitoyl cardiolipin and palmitoyl phosphatidyldiacylglycerol exhibit the characteristic multilamellar liposomal structure (Fig. 2). The vesicles are generally surrounded by 6—10 lamellae and are typically 2—20 μm in diameter. Vesicles of phosphatidyldiacylglycerol appear to be somewhat smaller than those of cardiolipin. This could be a manifestation of the smaller polar head group in the phosphatidyldiacylglycerol, which should give rise to a more curved surface in its vesicles.

Thermotropic behavior of the dispersions

Differential scanning calorimetric profiles of aqueous dispersions (>90% water by weight) of palmitoyl cardiolipin · 2 NH_4^+ and palmitoyl phosphatidyldiacylglycerol · Na^+ are shown in Fig. 3. Both of these lipids exhibit an endothermic phase transition with a characteristic transition temperature (T_m), enthalpy of transition (ΔH), temperature range of the transition, and size of the cooperative unit (n) undergoing the transition. No such thermotropic transitions are observed when the dry (i.e. containing 1—4 H_2O molecules of hydration) phospholipid samples are scanned. This suggests that the thermotropic transition is indeed a characteristic of bilayer organization assumed by the hydrated cardiolipin and phosphatidyldiacylglycerol salts. Such behavior is typical of all the natural phospholipids so far examined [25,26].



2A



2B

Fig. 2. Electron micrographs of negatively stained liposomes: (A) palmitoyl cardiolipin and (B) palmitoyl-3,3-phosphatidyldiacylglycerol. Liposomes were diluted with 0.5% phosphotungstic acid (pH 7.0) and placed on Formvar-coated EM grids. Scale marker = 60 nm (X182 000). The presence of 50 mM Ca^{2+} in the staining medium had no effect on the appearance of the liposomes.

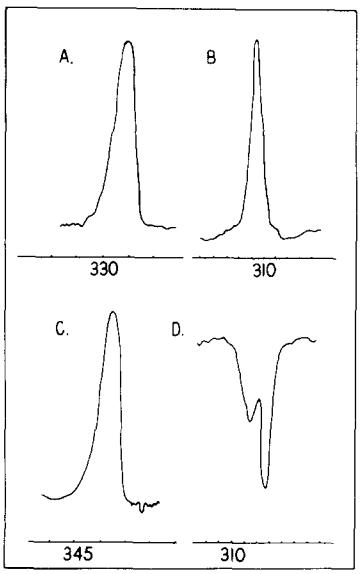


Fig. 3. Differential scanning calorimetric profiles of liposomes: (A) palmitoyl cardiolipin · $2NH_4^+$; (B) and (D) up and down scans of palmitoyl cardiolipin · $2Rb^+$; (C) palmitoyl-3,3-phosphatidyldiacylglycerol · Na^+.

Phase transition characteristics of dispersions of homologous cardiolipin and phosphatidyldiacylglycerol

The thermally induced phase transitions in phospholipid bilayers are known to involve a change in the state of the polymethylene chains in the bilayer. The transition characteristics depend upon both the length and degree of unsaturation of the hydrocarbon chain and the nature of the polar head group. We examined the effect of varying the acyl chain length on the thermotropic phase transition characteristics of homologous cardiolipin and phosphatidyldiacylglycerol. The data are summarized in Table I. The phase transition profiles of these dispersions have some features in common: the profiles are slightly asymmetric, and T_m for the heating scan is generally about 1—2°C above T_m for the cooling scan. As shown in Table I, the phase transition temperature and the enthalpies of transition increase monotonically with chain-length in the cardiolipins and the phosphatidyldiacylglycerol series. For a given chain-length T_m and the enthalpy of transition decrease in the order: phosphatidyldiacylglycerol > cardiolipin ~ phosphatidylcholine; the latter phospholipid has two acyl chains. An approx. 15 K change in T_m per $-CH_2-CH_2-$ residue is observed for both cardiolipin and phosphatidyldiacylglycerol. Such dependence of T_m upon chain-length has been observed for diacylphospholipids [25,26].

The average increase in enthalpy per methylene group is approximately twice as large among sodium salts of phosphatidyldiacylglycerol than among ammonium salts of cardiolipin. This supports the view that ΔH, like T_m, reflects changes in chain packing, and that the packing is affected by changes in both chain length and size of the polar head group. The values of the size of the cooperative units (n) calculated [27,28] for the various phospholipids from

TABLE I

THERMOTROPIC PHASE TRANSITION CHARACTERISTICS OF CARDIOLIPIN AND PHOSPHATIDYLDIACYLGLYCEROL

L = lauroyl; M = myristoyl; P = palmitoyl.

Phospholipid	T_m (K)	Enthalpy kcal/mol		Remarks
		Major	Minor	
P-Phosphatidylcholine	314.3	9.1	—	
Cardiolipin				
L · $2NH_4^+$	298.5	8.7	9	
M · $2NH_4^+$	313.0	12.5	—	
P · $2NH_4^+$	331.0	17.7	—	
P · $2Li^+$	315.5	15.2	12.7	Peak at 322°C
P · $2Na^+$	312.7	8.9	—	Shoulder
P · $2K^+$	327.5	12.4	5.7	Peak at 325 *
P · $2Rb^+$	311.5	14.1	11.5	Peak at 312 *
P · $2Cs^+$	325.5	23.9	—	
P · $2Ag^+$	324.5	13.0	7.0	Peak at 321
P · Mg^{2+}	344.0	30.5	—	
P · Ca^{2+}	361.5	24.2	—	
P ·Ba^{2+}	331.5	26.5	—	Broad split peak
P · Mn^{2+}	358.5	29.2	—	
P · Co^{2+}	324.5	14.7	—	
P · Zn^{2+}	340.0	17.5	—	
P · Cd^{2+}	351.0	35.4	—	
P · Pb^{2+}	341.5	21.0	—	
Spermine 1/1	332.5	10.4	—	
Phosphatidyldiacylglycerol				
L-3,3 · Na^+	325.3	10.2	—	
M-3,3 · Na	334.4	17.7	—	
P-3,3 ··Na^+	346.5	22.2	—	
P-3,3 · K^+	348.0	19.6	—	
P-3,3 · Mg $^{2+}$/2	335.3	23.2	—	
P-3,3 · Ca $^{2+}$/2	326.1	—		
P-3,3 · Cd $^{2+}$/2	329.7	27.4	—	
P-3,3 · Mn $^{2+}$/2	329.0	27.7	—	
P-1,1 · Na^+	344.9	23.4	—	
P-3,1 · Na^+ **	346.5	26.2	—	
P-1,3 · Na^+ **	346.1	27.5	—	

* Observed only on cooling scan.

** Same compound made by different phosphorylation sequences.

their transition profiles are between 50 and 100. Since it is known [29] that the value of n is very sensitive to trace impurities (<1%), it is not possible to attach much significance to these numbers.

Phase transition characteristics of stereoisomeric palmitoyl phosphatidyldiacylglycerol

The two chiral centers on the phosphatidyldiacylglycerol molecule give rise to three stereoisomers (Fig. 1B). Some of these isomers could show difference in packing characteristics in the bilayer that would be reflected in their phase transition characteristics. The data in Table I show that all the transition parameters for the three stereoisomers are identical within experimental error.

The 1,1-isomer shows a slightly lower T_m (~1.5°C) even though all samples were prepared with the same batch of fatty acids and by the same synthetic and purification procedures. Although our calorimetric techniques allow reproducibility to better than ±0.5°C, we cannot attribute significance to such small differences in T_m between stereoisomers, and believe that they arise from traces of impurity that are not detected by TLC.

Our observations on the thermotropic phase transition behavior of stereoisomeric phosphatidyldiacylglycerol do not conform to the conclusion put forward by Arnett et al. [30] based on studies of non-phospholipids in monolayers on 6 N H_2SO_4 subphase. It may be noted, however, that our respective amphipaths and techniques to study packing are markedly different.

Phase transition characteristics of dispersions of metal ion cardiolipin and phosphatidyldiacylglycerol salts

The phase transition characteristics of aqueous dispersions of cardiolipin and phosphatidyldiacylglycerol metal salts differ significantly from those of the ammonium salt. Moreover, in cardiolipin the T_m for divalent metal salts are consistently higher than those for monovalent metal salts. For one trivalent metal salt studied, Sc^{3+}, the T_m appears to be above the boiling point of water.

Fig. 4 shows plots of T_m vs. unhydrated ionic radii [31] for mono- and divalent metal salts of cardiolipin. Among divalent ions, the Ca^{2+} salt exhibits the highest T_m; T_m decreases with both an increase and a decrease in ion radius. Among monovalent ions, the biphasic dependence of T_m on unhydrated ionic radius is less pronounced, with K^+ and Ag^+ at the peak of the curve. One interpretation of these types of curves is that better interaction among acyl chains is in general achieved in the divalent metal salts, and that optimum interchain interaction occurs at a radius of about 0.9 Å for divalent ions, and at about

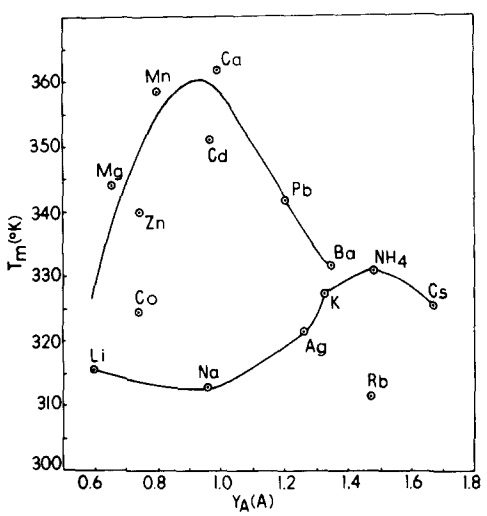


Fig. 4. Plot of T_m for liposomes of palmitoyl cardiolipin salts as a function of unhydrated ionic radii of cations. The mono- and divalent cations appear to fall on distinct curves.

1.4 Å for monovalent ions, irrespective of their degree of hydration in the salts in their aqueous dispersion.

Two salts were made from different stoichiometric proportions of cardiolipin and spermine, and their T_m values are somewhat higher than that for the ammonium salt.

Most alkali metal salts of cardiolipin exhibit two transition peaks of the type shown in Fig. 3 for the Rb salt. We attribute the larger of the two peaks to the normal gel-to-liquid crystalline thermotropic phase transition, which presumably arises from conformational reorganization of the acyl chains. The minor peaks occur at lower or higher temperature relative to the main transitions, depending on the metal. The minor peak is generally observed in the cooling temperature scan compared to the heating scan. The enthalpy of the minor peak is generally from 10 to 30% that of the major peak. This dual-transition phenomenon is not observed with divalent metal salts or with Na^+ phosphatidyldiacylglycerol salts, and we have not detected it either with Cs^+ or NH_4^+ cardiolipin. A possible explanation for the appearance of a minor peak is that it may result from a conformational reorientation of the polar head group of cardiolipin that is associated with a monovalent metal ion. This reorientation may precede or follow the main transition of the acyl chains. We suspect that in Cs^+ and NH_4^+ cardiolipin the two transitions are too close for resolution.

The transition enthalpies of the cardiolipin salts vary from 8.9 kcal/mol for the sodium salt to 35.4 kcal/mol for the cadmium salt, with the divalent metal salts tending, in general toward higher values. These larger enthalpies could reflect the occurrence of intermolecular polar head group interactions in cardiolipin due to bridging of phosphate groups by divalent cations.

Among salts of phosphatidyldiacylglycerol, the T_m values for divalent metal salts are lower than those for monovalent metal ions. This trend is opposite to that observed among the respective metal ion salts of cardiolipin.

Discussion

The results of this investigation show that the thermotropic phase transition properties of tetraacyl acidic phospholipids are influenced by the length of the acyl chains, and by the charge-type and the radius of the cations that are bound to the polar head group. The response of T_m and ΔH to changes in acyl chain length are virtually identical among diacyl [25,26] and tetraacyl phospholipids; therefore, we conclude that analogous forces regulate the interactions between acyl chains among the two types of phospholipids.

Concerning the effects of cations on the phase transition characteristics of the two types of acidic tetraacyl phospholipids, we attribute these effects to modulation of the polar head group by the cations. In general, three factors could contribute to this modulation of the polar head group resulting in changes in acyl chain interactions: (a) size of the head group, (b) charge type of the head group, and (c) charge type and radius of the countercation bound to the head group. These factors could alter the conformation of the glycerol and the phosphate moieties of the polar head group. These conformational changes may, ultimately, lead to alterations in interchain separation and therefore in the degrees of freedom of the chains. Changes in glycerol conformation are

perhaps responsihle for the 'pretransitions' that are observed at 5—15°C below the main transition temperature, with about 10% of the main transition enthalpy, in most phospholipids including the ones reported here. We propose that changes in the orientation of phosphate groups, and hence changes in the effective size of the polar head group, may be responsible for the dependence of the bilayer phase properties on the nature of the cation and on the number of phosphate groups in the polar head of cardiolipin and phosphatidyldiacylglycerol. Fig. 5 shows a molecular model of the 'compact' conformation of cardiolipin. A similar model showing a smaller polar head group can be constructed for phosphatidyldiacylglycerol. The larger head group may result in larger separation among neighboring acyl chains and hence in lower van der Waals interactions and lower T_m for the former vs. the latter phospholipid type. In cardiolipin, a divalent cation could bind the two phosphates intramolecularly, but in phosphatidyldiacylglycerol the divalent cations can only bind phosphate intermolecularly.

The above picture may also account for the appearance of a second transition in the calorimetric profiles of monovalent (but not of divalent) cation salts of cardiolipin. Thus, the divalent cations may lock the two phosphate groups and prevent reorientation of the polar head group. Monovalent cations may give rise to different conformational states of the polar head group associated with different acyl chain interactions.

Monolayer studies (soon to be published) show that the molecular area/acyl chain in both diacyl and tetraacyl phospholipids are identical and their cross-sectional areas are 4 times that of palmitic acid. This supports the compact conformation of cardiolipin (Fig. 5). Similarly, the molecular areas of cardiolipin and phosphatidyldiacylglycerol are found to be identical, both in the absence and in the presence of 50 mM Ca^{2+} in the medium. Apparently, the differences noted for these phospholipids in monolayer and bilayer studies reflect the additional factors which regulate the packing of the phospholipids in the bilayer.

Finally, it is known that aqueous dispersions of cardiolipin [32], like those of other phospholipids [4,25,26], give rise to extensive polymorphism at various water to phospholipid ratios. However, at the ratios utilized in the present

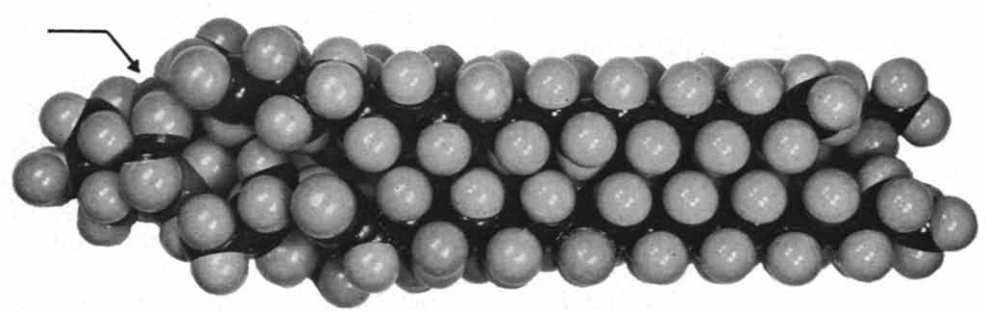


Fig. 5. Compact conformation of the cardiolipin dianion. Arrow points to the binding site of metal ions, 2 M^+ or M^{2+}.

work, the predominant phase is probably lamellar as suggested by the electron micrographs. This is further supported by the sharpness of the transition, and its dependence on chain-length as has been observed for other phospholipids [25,26].

The dependence of the cardiolipin phase transition temperature on the cation radius suggests that there is an optimum size of metal ion for optimal packing of the acyl chains in the bilayers. It may not be coincidental that two of the most important ions involved in mitochondrial membrane function, Ca^{2+} and K^+, lie on the peak of the respective biphasic curves. Cardiolipin is a major constituent of the inner mitochondrial membrane, and it may be that without the participation of Ca^{2+} or K^+, the phase transition of the bilayer resulting from the highly unsaturated cardiolipin in the mitochondria would be lower than their normal ambient temperature. Thus, a 40°C increase in transition temperature induced by Ca^{2+} could have significant regulatory effect on the function of mitochondrial membrane proteins.

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