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PHOSPHATIDYLCHOLESTEROL BILAYERS

A MODEL FOR PHOSPHOLIPID-CHOLESTEROL INTERACTION

MAHENDRA K. JAIN [a], FAUSTO RAMIREZ [b,*], TERENCE M. McCAFFREY [b], PANAYIOTIS V. IOANNOU [b], JAMES F. MARECEK [b] and J. LEUNISSEN-BIJVELT [c]

[a] *Department of Chemistry, University of Delaware, Newark, DE 19711,* [b] *State University of New York, Stony Brook, NY 11794 (U.S.A.) and* [c] *Department of Cell and Molecular Biology, The State University, Utrecht (The Netherlands)*

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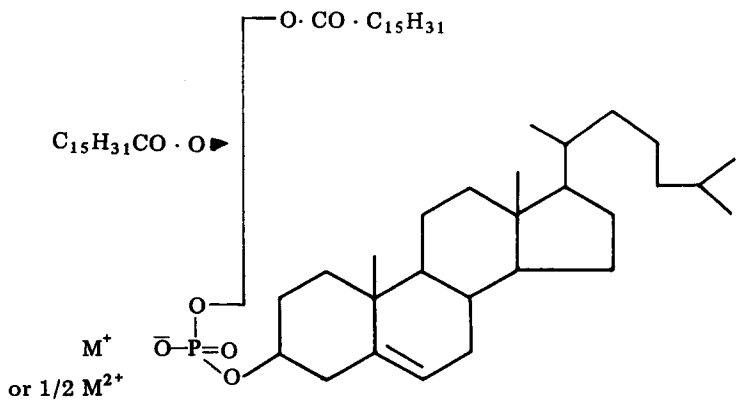
Summary

Aqueous dispersions of monovalent and divalent cation salts of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol form multilamellar vesicles as shown by freeze-fracture electron microscopy, by electron micrographs of the negatively stained liposomes, and by swelling curves of liposomes in hypoosmotic medium. Differential scanning calorimetry reveals that aqueous dispersions of divalent metal salts of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)-cholesterol undergo a characteristic thermotropic phase transition with a relatively large cooperative unit ($n > 250$ for the calcium salt). In contrast, monovalent cation salts of *O*-(1,2-dipalmitoyl-*sn*-glycerol-3-phosphoryl)cholesterol do not show a thermotropic phase transition under comparable conditions. The molecular area of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol in a monolayer is the same in the presence and absence of Ca^{2+}, and is virtually equal to the area of an equimolar mixture of dipalmitoyl phosphatidic acid and cholesterol. To account for the novel state induced by Ca^{2+} on aqueous dispersions of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol (i.e., bilayer organization and highly cooperative phase transition), a linear array model is proposed in which Ca^{2+} bridges adjacent arrays of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol molecules, thus freezing the acyl chains in their normal state. One of the main corollaries of the model is that the cooperative unit for a thermotropic phase transition is essentially one-dimensional, rather than a two-dimensional matrix. *O*-(1,2-Dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol is proposed as an orientationally and conformationally restricted analog of glycerophospholipid plus cholesterol in bilayers.

Introduction

The state of cholesterol in biological membranes and phospholipid bilayers is not yet understood [1,2]. There is general agreement that cholesterol is anisotropically oriented parallel to phospholipid acyl chains with the 3β-OH group near the glycerol backbone, and that the sterol restricts freedom of motion of the acyl chains. The addition of less than 25 mol% of cholesterol to certain saturated phospholipids results in the separation of a sterol-deficient phase from a sterol-rich phase [3,4]. In such systems, the gross bilayer organization is retained only up to a maximum incorporation of 50 mol% of cholesterol. A number of 3β-sterols have been incorporated into phospholipid bilayers, and in such systems the permeability characteristics of the resulting liposomes depend on the molecular structure of the sterol and on its mole percent in the mixture. In general, the gel-to-liquid crystalline phase transition of saturated phospholipids disappears at or below approx. 50 mol% incorporation of sterol. Ether or hydrocarbon analogs of phosphatidylcholine give rise to bilayers which are also susceptible to phase separation upon addition of less than 25 mol% of cholesterol (Jain, M.K., Ramirez, F., McCaffrey, T.M., Ioannou, P.V. and Marecek, J.F., unpublished results). There is at present no clear understanding of the mode of interaction of cholesterol or of other sterols with diacylglycerophospholipids or their analogs.

A recent paper [5] described the synthesis of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol, abbreviated hereinafter as phosphatidylcholesterol. A racemic form of the same type of compound has also been prepared by a different route [6]. The optically active *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol can be regarded as an orientationally and conformationally restricted analog of a glycerophospholipid plus cholesterol in bilayers. The present work shows that aqueous dispersions of monovalent and divalent cation salts of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol form osmotically intact multilamellar vesicles. However, only the dispersions of divalent metal salts of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol give detectable peaks in differential scanning calorimetry, indicative of thermotropic phase transitions with relatively high cooperativity. These observations



DPCh

suggest that the two acyl chains of a diacylglycerophosphoryl moiety can interact with one cholesterol residue to generate a structure capable of participating in the type of intermolecular interactions which give rise to gel-to-liquid crystalline transitions of relatively high cooperativity. The synthetic procedure which gives *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol in good yield fails to produce detectable amounts of the isomer derived from epicholesterol, emphasizing the overriding geometrical and steric factors which operate in these phosphatidylsterol compounds.

Materials and Methods

Synthesis of O-(1,2-Dipalmitoyl-sn-glycero-3-phosphoryl)cholesterol. The calcium, manganese(II) and ammonium salts of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol, $C_{62}H_{112}O_8P \cdot 0.5\ Ca \cdot H_2O$, $C_{62}H_{112}O_8P \cdot 0.5\ Mn \cdot 0.5\ H_2O$ and $C_{62}H_{112}O_8P \cdot NH_4 \cdot H_2O$, were synthesized as previously described [5]. The samples gave one spot, $R_f = 0.25$, in thin-layer chromatography on 5 × 20 cm silica gel plates (Merck No. 7736) using chloroform/methanol/conc. NH_4OH (30 : 5 : 1, v/v). The new magnesium, lithium, sodium and potassium salts were prepared from the calcium salts as previously described for the manganese salt [5] but using 2 M aqueous solutions of $MgCl_2$, LiCl or NaCl, or 1.5 M aqueous solution of KCl, respectively. Sample purity was established by thin-layer chromatography and elemental analysis:

$C_{62}H_{112}O_8P \cdot 0.5\ Mg$.
Calcd.: C, 72.4; H, 10.9; Mg, 1.2; M_r 1028.7.
Found: C, 72.3; H, 10.6; Mg. 1.1.

$C_{62}H_{112}O_8PLi$.
Calcd.: 72.7; H, 11.0; Li, 0.7; M_r 1023.4.
Found: C, 72.2; H, 11.2; Li, 0.7.

$C_{62}H_{112}O_8PNa$.
Calcd.: C, 71.6; H, 10.9; Na, 2.2; M_r 1039.5.
Found: C, 71.5; H, 11.2; Na, 2.2.

$C_{62}H_{112}O_8PK \cdot H_2O$.
Calcd.: C, 69.2; H, 10.7; K, 3.6; M_r 1055.6.
Found: C, 69.4; H, 11.3; K, 4.0.

These *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol salts were synthesized from palmitoyl chloride purchased from Sigma Chemical Co. (Grade I). A sample of the chloride was converted to the methyl ester by treatment with methanol. Analysis by gas chromatography showed it to be 99.0% methyl palmitate, containing 0.5% each of methyl penta- and heptadecanoates and traces (less than 0.05%) of methyl myristate and stearate. The compounds were identified by comparison of their chromatographic retention times with those of authentic samples of the esters. The gas chromatographic analyses were performed using a Hewlett-Packard 5830A gas-liquid chromatograph and the following operating conditions. Column: 6 ft × 1/8 inch 10% Apiezon L on Chromosorb W-H.P. Temperature: 245° C (isothermal). Carrier: He at 30 cm^3/min; detector: thermal conductivity at 275° C.

The synthesis of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol [5]

was repeated on a smaller scale from palmitic acid purchased from Nu Chek Prep, Inc. A sample of the acid in hexane was converted to the methyl ester by treatment with diazomethane. Gas chromatographic analysis using the conditions previously described was unable to detect any impurities showing that the acid was at least 99.9% pure. The acid was converted into the acid chloride and to the diglyceride as described [5].

The formulae given for the ammonium and the metal salts of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol are derived from elemental analysis carried out on samples that had been kept 24 h at 25° C and 0.1 Torr. The water content of several of the samples (Ca, Mn, NH_4) was confirmed by direct K. Fischer determination (performed by Galbraith Laboratories, Knoxville, TN). The samples were stored in a cold, anhydrous environment prior to the preparation of their aqueous dispersions. The ammonium and the mono- and divalent metal salts of phosphatidylcholesterol readily absorb water at ambient temperature. Less than 2% of hydrolytic decomposition was observed when these samples were kept for approx. 30 min at 80° C. Thin-layer chromatography showed that this small amount of hydrolysis generates cholesteryl-phosphate rather than phosphatidic acid.

Analytical techniques. Vesicles were prepared by dispersing a film of appropriate lipid(s) in water or in salt solution, with the aid of a vortex mixer, for approx. 1 h at 60° C [7]. Swelling of liposomes in response to hypo-osmotic shock was monitored at 360 nm on a Cary spectrophotometer [8]. Freeze-fracture electron microscopy was performed according to well-established techniques [9]. Calorimetric scans were performed with a Perkin Elmer DSC-1B instrument at 0.6—5 K per min at highest sensitivity [10]. To a known amount (approx. 1 mg) of the lipid, 10 µl of distilled water were added, and the aluminium sample pan for differential scanning calorimetry was sealed. The pan was then heated to 345 K and allowed to equilibrate for 10 min in the sample compartment. The samples were usually cooled to below 300 K and then scanned on heating and cooling cycles.

The pressure-area isotherms at the air/water interface were measured on a Langmuir through which was calibrated with stearic acid [11].

Results and Discussion

Aqueous dispersions of monovalent and divalent metal salts of phosphatidylcholesterol form multilamellar vesicles. Fig. 1 shows the freeze-fracture faces obtained on one of these dispersions in the absence of divalent cations, and it is apparent that closed vesicular structures are present in the dispersion. Similar results are obtained in the presence of Ca^{2+}. The fracture planes suggest the presence of bilayer orientation. The size of the liposomes, 5000 Å in diameter, is virtually the same as that obtained from electron micrographs (not reproduced) of liposomes made from the ammonium or calcium salt of phosphatidylcholesterol which had been stained with 1% phosphotungstic acid at pH 7.4 (134 000 ×, magnification).

Vesicles made from phosphatidylcholesterol in high-salt solution, in the presence or in the absence of Ca^{2+}, exhibit osmotic swelling when placed in low-salt solution (Fig. 2). A similar phenomenon is observed with an equimolar mixture of dipalmitoyl phosphatidic acid plus cholesterol. The swelling charac-

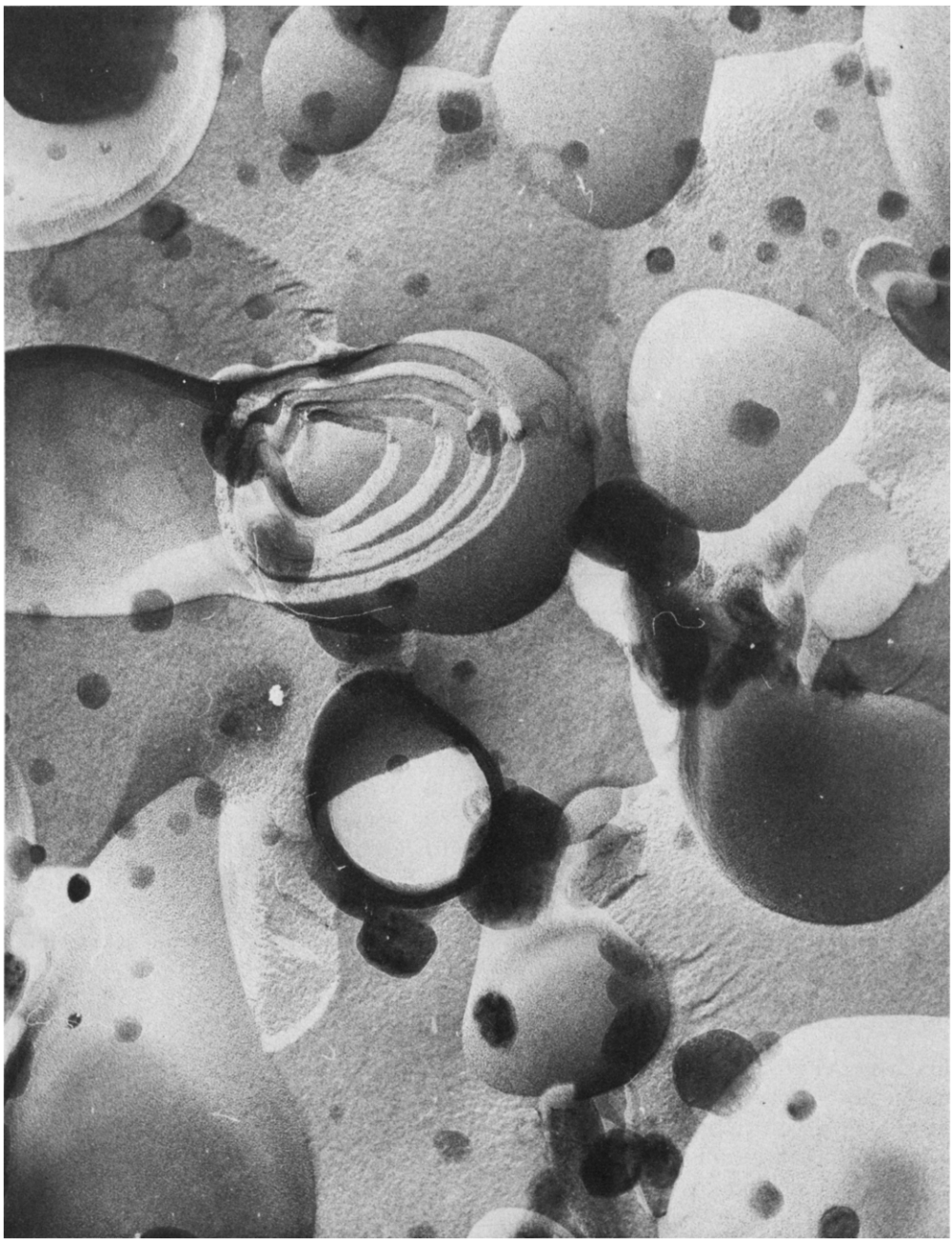


Fig. 1. Electron micrograph of the freeze-fracture replica of dispersions of dipalmitoylphosphatidylcholesterol. (a) Acid form, (b) calcium salt.

teristics of phosphatidic acid are quite different in the absence and in the presence of cholesterol.

The thermal behavior of aqueous dispersions of phosphatidylcholesterol monovalent and divalent salts was studied by means of differential scanning

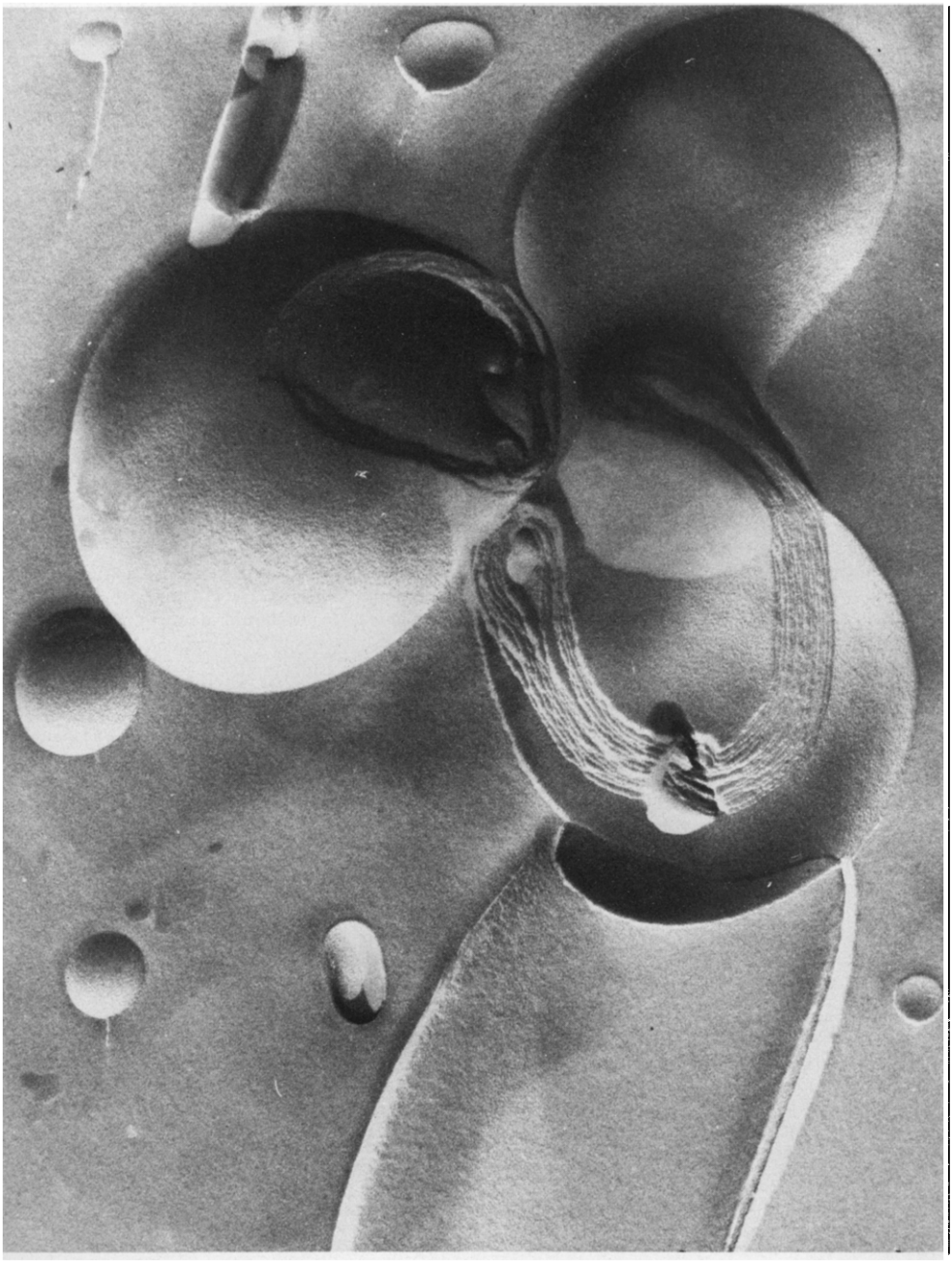
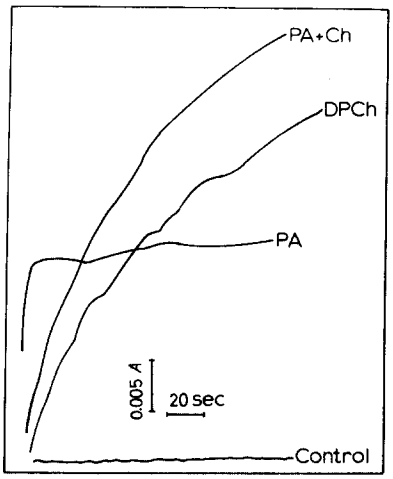


Fig. 1b. Magnification, ×76 000.



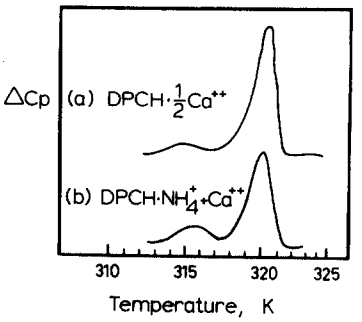


Fig. 2. Swelling rates of liposomes in response to hypo-osmotic shock. Liposomes were prepared in 100 mM KCl/10 mM Tris buffer at pH 7.4. Rates of changes in transmitted light scattering at 360 nm were followed at 22°C in samples made from 20 μl of the liposomes (10 mg lipid/ml) and 2.5 ml of water. The control trace (no change in light scattering) refers to liposomes observed in an iso-osmolar salts solution. *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol, dipalmitoyl phosphatidylcholesterol (this work). The same swelling rate is observed with liposomes made from *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol · 1/2 Ca^{2+}, in the absence or in the presence of 1 mM ethylenediaminetetraacetic acid, and from *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol · NH_4^+, with or without added 20 mM $CaCl_2$. PA, dipalmitoyl phosphatidic acid (Sigma Chemical Co. optimum grade); Ch, recrystallized cholesterol. DPCh, dipalmitoyl phosphatidylcholesterol.

Fig. 3. Differential scanning calorimetric profiles of water dispersions. *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol · 1/2 Ca^{2+}, preformed calcium salt of dipalmitoyl phosphatidylcholesterol. *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol · NH_4^+ + Ca^{2+}, preformed ammonium salt of dipalmitoyl phosphatidylcholesterol with 100 mM added $CaCl_2$. Same profile is obtained on repeated scans of same sample, with or without sample equilibration (5 min, 350 K) prior to scan. Lipid is recovered unchanged after scans. No peak is detectable upon addition of ethylenediaminetetraacetic acid (50 mM) to *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol · 1/2 Ca^{2+} dispersion, or from *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol · NH_4^+ in the absence of $CaCl_2$. DPCH, dipalmitoyl phosphatidylcholesterol.

TABLE I

THERMOTROPIC PHASE TRANSITION CHARACTERISTICS OF DIPALMITOYL PHOSPHATIDYLCHOLESTEROL SALTS IN AQUEOUS DISPERSIONS

The salt (1 mg) was allowed to swell in distilled water (10 μl) and the differential calorimetric scans were carried out as described in the text. The 1,2-diacyl-*sn*-glycerol used in the synthesis was made from palmitoyl chloride of 99.0% purity. DPPC, dipamitoyl phosphatidylcholine; PA, dipalmitoyl phosphatidic acid.

Salt	Major peak			Minor peak	
	T_m (K)	ΔH (kcal/mol)	n	T_m	ΔH
1/2 Mg^{2+}	328.8	6.0	52	316	0.6
1/2 Ca^{2+}	320.0	8.0	64 *	315	0.8
1/2 Mn^{2+}	316.3	10.8	50	310	1.2
DPPC	314.5	9.1	230	306	1.0
PA	340.0	5.7	180	336	0.4

* A value of $n \geqslant 250$ was observed when the 1,2-diacyl-*sn*-glycerol was made from palmitic acid of 99.9% purity. Other transition parameters for this sample remained virtually unchanged.

calorimetry. Dispersions of the NH_4^+, Li^+, Na^+, and K^+ salts do not give a detectable thermotropic phase transition from 250 to 360 K. However, in striking contrast, the Mg^{2+}, Ca^{2+} and Mn^{2+} salts exhibit a characteristic thermotropic phase transition. The results are summarized in Table I and Fig. 3. The mid-point transition temperatures (T_m) for the heating scans are about 2 K above T_m for the cooling scan. The scans show a major and a minor peak. We attribute the larger of the two peaks to the normal gel-to-liquid crystalline transition which presumably arises from conformational reorganization of the acyl chains. T_m values for the minor peaks are lower than those of the major peaks, and the minor transition enthalpies are approx. 10% of the major transition enthalpies. Minor 'pretransitions' of this type are observed in many phospholipids, and are probably associated with changes in conformation of the glycerol moiety of the polar head group, which may ultimately lead to alterations in interchain separation and changes in degrees of freedom of the chains.

The size of the cooperative unit, n, for the phase transition of phosphatidylcholesterol divalent salts is calculated from the following expression [12,13]:

$$n = \frac{4R\ \Delta C_p T_m^2}{\Delta H^2}$$

The values of n given in Table I were obtained on samples made from palmitoyl chloride of 99.0% purity. In view of the sensitivity of n to trace amounts of impurities [14] we carried out a small-scale synthesis of phosphatidylcholesterol using as starting material palmitic acid of 99.9% purity. The resulting sample furnished a calcium salt of which the aqueous dispersions showed a phase transition with a significantly larger cooperative unit ($n > 250$), although other transition parameters remained virtually unchanged.

The thermotropic phase transition characteristics of divalent metal salts of phosphatidylcholesterol are analogous to those previously observed for dipalmitoyl phosphatidylcholine [3,4] and dipalmitoyl phosphatidic acid [15], all in aqueous dispersions. Thus, it appears that, under certain circumstances, the phosphatidylcholesterol molecules in water are indeed able to form a gel phase that undergoes a highly cooperative transition to a liquid crystalline phase. It is not entirely ruled out that the monovalent cation salts of phosphatidylcholesterol give rise to phase transitions of low cooperativity, and hence produce very broad peaks which are undetectable by our experimental techniques. Experiments with the ammonium salts in 30% ethyleneglycol disclosed no peak under the peak for melting ice.

It was of interest to compare the molecular area of phosphatidylcholesterol with that of an equimolar mixture of dipalmitoyl phosphatidic acid plus cholesterol. The surface pressure-area relationships of these systems in monolayers, at the air/water interface are shown in Fig. 4. The molecular area of phosphatidylcholesterol, 85 Å^2, is quite close to the sum of the areas of phosphatidic acid, 45 Å^2, and cholesterol, 39 Å^2. The molecular area of an equimolar mixture of phosphatidic acid plus cholesterol is also quite similar, 79 Å^2 (the coefficient of variation is ±5%). These phospholipid monolayers are more compressible below 25 dyne · cm^{-1}, and the collapse pressure of the films is approx. 50 dyne · cm^{-1}. Thus, phosphatidylcholesterol monolayers behave like those of other phospholipids [16].

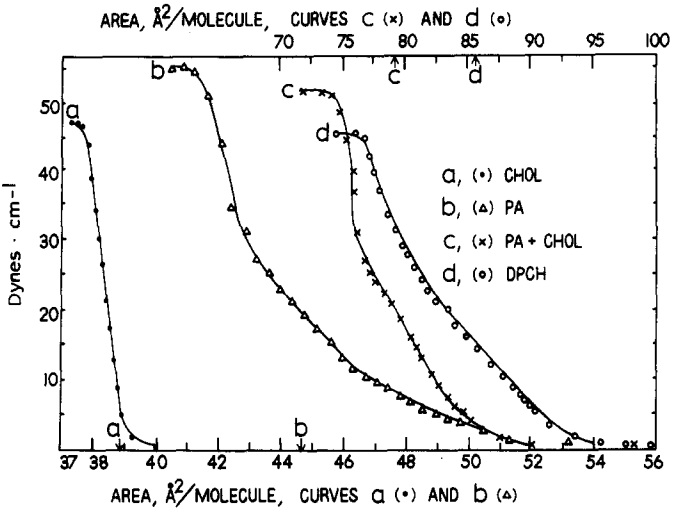


Fig. 4. Pressure-area isotherms at 22°C. (a) Chol, cholesterol; (b) PA, dipalmitoyl phosphatidic acid; (c) equimolar PA + Chol; (3) *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol, dipalmitoyl phosphatidylcholesterol · NH_4^+ (DPCH). Subphase: 10 mM $NaHCO_3$ at pH 8.4 in all cases. The area/molecule is not affected by the presence of Ca^{2+} in the aqueous phase. Arrows indicate the molecular areas at zero pressure.

The main results of the present investigation are. (1) The formation of osmotically active liposomes in aqueous dispersions of phosphatidylcholesterol. (2) The observation that divalent metal ions, in particular Ca^{2+}, induce a novel state in the aqueous dispersions of phosphatidylcholesterol, and this state undergoes a cooperative phase transition detectable in differential scanning calorimetry suggesting extended acyl-acyl chain interactions. To rationalize these observations, we propose the following model.

An examination of scalar molecular models of dipalmitoyl phosphatidylcholesterol reveals that appropriate rotations around the set of bonds a—d of the polar head group,

(glycerol) C3$\overset{a}{-}$O$\overset{b}{-}$P$\overset{c}{-}$O$\overset{d}{-}$C3′(cholesterol) ,

results in the folding of the cholesterol moiety on the two acyl chains, with the relatively flat α-face of the sterol in contact with the acyl chains. This parallel alignment of cholesterol and acyl chains, with the chains in the all-*trans* conformation, constitutes a nearly cylindrical and compact structure suitable for the type of van der Waal's interactions which are required for the formation of a two-dimensional matrix. In essence, it is suggested that dipalmitoyl phosphatidylcholesterol molecules form a two-dimensional matrix with the glycerophosphate groups in the aqueous phase and the hydrophobic acyl chains and the sterol nucleus in the interior of the bilayer. The arrangement of the *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol molecules in the bilayer matrix is such that the acyl chains form an uninterrupted linear array, which is separated from another acyl chain array by an array of cholesterol nuclei. In a recent paper, Rogers et al. [17] have suggested an analogous type of linear array with uninterrupted acyl-acyl chain interactions to account for the behav-

ious of 1 : 1 mixtures of phosphatidylcholine and certain sterols. These authors [17] have discussed two of the several possible arrangements that can give rise to such linear arrangements.

In the model for aqueous dispersions of dipalmitoyl phosphatidylcholesterol proposed here, the linear arrays of acyl chains are separated by about 10 Å. This would be an optimal separation for the bridging of adjacent parallel chain arrays by divalent metal ions. Mg^{2+} [18] and Ca^{2+} [19] are known to engage in tight binding with oxyanions of phosphodiesters. Therefore, we suggest that, in the two-dimensional bilayer matrix, the arrays are stabilized by acyl-acyl chain interactions in one dimension, and by electrostatic metal ion-phosphate bridges in the second dimension. The divalent cation, essentially, restricts the free rotation of *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)-cholesterol molecules, thus enhancing acyl chain interactions over the length of an acyl chain array. The monovalent metal salts of *O*-(1,2-dipalmitoyl-*sn*-glycerol-3-phosphoryl)cholesterol would have a relatively free rotation around their long axis, which should disrupt acyl chain interactions.

This model accounts for the appearance of a cooperative phase transition in Ca^{2+}-*O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol bilayers, and in other divalent cation-*O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)cholesterol bilayers. The model is also consistent with the magnitude of the enthalphy change associated with a full overlap of palmitoyl chains. The model leads to a novel interpretation for the cooperativity of phase transition, in general. In the present context, we postulate that the acyl chains which undergo transition are in linear arrays. Although the concepts associated with phase transition in bilayer have been developed for a two-dimensional array of acyl chains, it does not necessarily follow that the cooperative units should be two-dimensional arrays, even in a symmetrical two-dimensional matrix. Indeed, long linear domains have been observed in the freeze-etched electron micrographs of phospholipid bilayers [20]. Molecules at the phase boundaries, i.e., at the edge of such domains as are being considered here, would be in relative disorder [21] compared to molecules in the second and subsequent layers away from the phase boundaries. Thus, phase transition may be visualized as a cooperative process in which one array of molecules at a time peels off from the phase boundaries. This occurs presumably because only those molecules at the phase boundary of a domain have an identical environment and a lower activation energy. Molecules in the middle of a domain are much more restricted. A finite width, and an intrinsically asymmetric shape of the phospholipid phase transition profiles [14] would be consistent with such cooperative 'peeling' as a general mode for phase transition. In summary, cooperativity of a phase transition may be a one-dimensional phenomenon occurring along the phase boundaries of a domain.

Acknowledgments

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