Transitions and molecular packing in highly purified 1,2dipalmitoyl phosphatidylcholine–water phases. I. Transitions, improved phase diagrams, method of packing analysis, molecular structures of diglyceride, and polar regions

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Citation: The Journal of Chemical Physics **78**, 4676 (1983); doi: 10.1063/1.445313
View online: http://dx.doi.org/10.1063/1.445313
View Table of Contents: http://scitation.aip.org/content/aip/journal/jcp/78/7?ver=pdfcov
Published by the AIP Publishing

Transitions and molecular packing in highly purified 1,2-dipalmitoyl-phosphatidylcholine–water phases. I. Transitions, improved phase diagrams, method of packing analysis, molecular structures of diglyceride, and polar regions

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(Received 15 February 1982; accepted 12 April 1982)

Results of extensive studies of phases prepared from highly purified 1,2-dipalmitoyl-*sn*-phosphatidylcholine and 1,2-dipalmitoyl-*sn*-glycerol are presented. The methods used included x-ray diffraction and thermal analysis and both temperature and water content were varied. Details of the many well-defined phases obtained, all with bilayer structures, are given in tables. An improved phase diagram was obtained from thermal data which showed well-defined transitions, and from discontinuities in a plot of bilayer repeat spacings with water content. For analysis of molecular packing the bilayer was divided into diglyceride and phosphorylcholine regions. The properties of the diglyceride regions of both compounds are discussed. Estimates of the size and shape of the polar groups are made and the general principles involved in their packing and the influence of water are discussed. Among new features reported, are the existence of the $0\bar{1}$ close chain packing for the crystalline dipalmitoyl glycerol but not in any lecithin phase. Three single crystal lecithin phases with different water contents were prepared which are more stable than the hexagonal chain phases usually reported in the same regions. These hexagonal phases slowly recrystallize to more stable forms, but are usually obtained on cooling melts or higher temperature phases. However, the single crystal phases only exist over restricted composition ranges while phases with hexagonal, square, and disordered chain packing can accommodate a variation in water content by a tilt of the chain axes and changes in bilayer thickness. Transition widths vary and are extremely sensitive to impurities.

INTRODUCTION

Phospholipids form many phases when heated or with changes in water content. The molecules tend to stack in bilayers because of their shape and give crystals or liquid crystals depending on the binding between the bilayers. Many of the liquid crystal phases are smectic and well ordered in the bilayer plane, as in crystals. At low water content, of up to > 5%, melting points exceed 200 °C, similar to ionic crystals. When the water content is higher, the bilayers become separated by water, loosely held by the polar groups. This is often as a uniform layer, but spherical shapes form readily under the influence of surface forces. In aqueous dispersions, a disordered chain region appears at temperatures which are slightly lower than those at which the corresponding diglyceride phase melts in the absence of water. Stable bilayers remain and this can be attributed primarily to the polar groups. In addition to their unusual physical and chemical properties, the phases formed in aqueous dispersions have structures common to biological membranes.

Study of phospholipids is difficult due to the complexity of the phase diagrams, problems in control of water content, and a sensitivity to trace impurities. Phase transitions may result from changes in water content or temperature and are broadened by impurities. The liquid crystal phases do not give x-ray data suitable for single crystal structural studies although valuable diffraction data can be obtained. These problems and others have led to substantial differences in experimental results and interpretations. This study of well defined phases prepared from highly purified materials resolves these difficulties and has allowed an analysis of the molecular packing in lecithin and lecithin–water phases to be made. This provides satisfactory structures for the polar regions in many phases for the first time and relates the structures of these bilayer phases to each other. The nature of the phase transitions has been the focus of considerable speculation despite the inadequacy of the experimental data in the absence of purified material and well define phases. Details of many of these transitions and the influence of intermolecular forces have now been clarified and will be described.

This article describes results obtained mainly by the study of highly purified phospholipids. Several single crystal phases were made and from them other phases prepared. A variety of physical methods were used and not all details can be given here. The purpose of these articles is to describe how physical properties are related to the structural chemistry of lecithin–water phases. The results, obtained with purified materials, provide a sound basis for further investigation of the influence of other molecules on the structures and transitions. This is of major physical and biological interest.

As three dimensional data is available for only a few lecithin–water phases, molecular packings are established by using the principles of crystal chemistry as employed by Pauling.[1]

For several phases, separate two dimensional subcells were identified in the polar and chain regions. Using standard molecular dimensions, stable arrays

 0021-9606/83/074676-11$2.10

of charged groups, as given by the packing analysis, were found to correspond with unit cell dimensions or molecular areas for all the phases studied. Only small changes in molecular conformation were required between the phases. In addition to forming a consistent picture for all the phases studied, the proposed structures were related in detail to a recent lecithin crystal structure.

The methods of packing analysis were originally developed to indicate the most probable structures for refinement by crystallographic analysis. They have been largely superseded by Patterson analysis, heavy atom substitution, and direct methods for samples available as single crystals. Crystal structure analysis cannot be applied directly to most DPPC phases. Agreement between predictions from the structures obtained by packing analysis and the observed data will indicate whether the structures are correct. The data for this is obtained by x-ray diffraction, thermal, spectroscopic, and other methods, including density measurements. The large number of phases formed by packing the same molecules provide convincing evidence for the structures proposed.

Analysis of packing in two and three dimensional systems; layer stacking, due to an elongated molecular shape, simplifies analysis of packing by reducing it to two dimensions on a molecular scale. However, more details of the molecular and atomic packing are required to understand the phase behavior, physical properties, and biological functions of phospholipids.

Other characteristics of phospholipids result from the presence of both polar and nonpolar groups in the molecule. Binding between groups of the same type is greatly favored, giving molecular bilayers and determining the influence of water on the structures. This again simplifies analysis of the atomic packing by allowing a division of the bilayer into regions according to the intermolecular forces. The atoms are packed in three dimensions, but the principles governing this packing are well established for each region.[1] In the polar region the largest intermolecular forces arise from the charged nitrogen and phosphate oxygen atoms. The tetrahedral binding of the phosphate and the nitrogen atom fix the geometry of these charged groups. These groups are linked by a flexible dimethylene–ester group allowing some variation in their packing and the ester–methylene group joining the polar and diglyceride regions allows differences in the packing of parts of the same molecule in the two regions.

Attachment of the polar groups to the diglyceride restricts their packing to two dimensional layers for which a packing analysis is simple when the bilayers are isolated, as in excess water. When the water content is low, a double polar layer forms and the packing is more complex.

In the diglyceride region, even when disordered, the chains are extended in a roughly cylindrical shape. This gives the most effective packing and despite the flexible bonds only small deviations from the extended conformation occur. The regular packing along the chain axes arises from the tetrahedral bonding of the carbon atoms. Lateral packing between the chains is closer for more regular stacking of the hydrogen atoms. An hexagonal close packing with chains of cylindrical shape, or closely related structures are common. The chains form three dimensional structures with the atoms packed into a series of subcells which can be readily identified.

In each carboxy–ester group the five atoms form planar rigid structures and the two carboxy–ester groups are joined by a single carbon to carbon bond. This considerably restricts the folding which is necessary to pack the chains effectively with their axes parallel. The motions of the atoms will be restricted in the carboxy–ester groups and their presence will result in a variation of mobility along the chains. The carboxy–ester groups form a significant part of bilayer structures, which has been neglected and comprise 20% of the number of non-hydrogen atoms in DPPC. For a given compound, changes in the folding of the diglyceride group are unlikely.

The coordination number, or number of atoms in close proximity, depends on the relative size of the atoms or groups being packed. In DPPC phases, in two dimensions, it is six for the chains, diglyceride groups and the phosphorylcholine groups. All form planar arrays with units of the same size. For packing the larger choline groups around single oxygen atoms, the ratio of the radii is 0.427 compared with the minimum of 0.414 required for octahedral packing in three dimensions with a coordination of 6. For ratios with intermediate values, the number is given by the next lower limit. An octahedral packing in three dimensions corresponds to a planar array with a coordination number of 4. Another bilayer can supply an additional choline group, bringing the number to 5. To pack two phosphate non-ester oxygen atoms, for the double volume, a mean radius and ratio of 0.638 are obtained, which give the same coordination as for a single atom. However, the ratio for packing choline and phosphate groups together has a maximum value of 0.884 and will certainly be larger than the 0.732 required for cubic body centered packing with a three dimensional coordination number of 8. This gives a polar region formed of a single layer of choline groups with another displaced layer of phosphate groups. The next layer of four choline groups for each phosphate will be absent because of the presence of the diglyceride groups. This is the same packing as calculated for the oxygen atoms alone. These coordination numbers apply to all DPPC and DPG phases including melts.

EXPERIMENTAL

Highly purified samples of 1, 2 dipalmitoyl-*sn*-phosphatidylcholine (DPPC) and 1, 2-*sn*-dipalmitoylglycerol (DPG) were prepared. A new approach was the use of sophisticated methods of solvent crystallization to give a large supply of excellent single crystals of three DPPC and one of the DPG phases. Methods of preparation and crystal growth have been reported.[2,3] The water content of the DPPC crystals was not, at first, es-

TABLE I. Data from single crystals and oriented samples of DPPC.

A. Crystals

Phase No.	H_2O (mol)	Space group	Unit cells (Å) all angles 90° a	b	c	Powder data; d (Å) index		Transition (°C)	Chain tilt (deg)
1	1	$P2_12_12$	97.0	10.80	8.90	X[a]	X	X	30
2	2	$P2_12_12_1$	8.776_6	8.953_7	119.95_8	3.887 4.489	213 020	63.2	12.5
3	3	$P2_12_12_1$	8.92 ±0.01	8.98 ±0.01	120.7 ±0.1	X	X	63	12

(B) Oriented samples

Phase no.	H_2O (mol)	Two-dimensional lattices all angles 90° a	b	Symmetry	Notes
4	0	6.56 8.48	6.56 4.90	square, polar groups hexagonal, chains	Axes parallel, three dimensional alignment at 50°C.
5	3–8	8.40	4.85	hexagonal, chains	Similar to $L\beta'$

[a] X, accurate parameters not available. The three possible relative alignments of the subcells in phase 4 are observed.

tablished with accuracy. High sensitivity calorimetric studies[4] indicated a purity greater than 99.94%. The single crystals are being studied by x-ray structural methods and details of the most accurate cell parameters available are given in Table I together with other data. These phases have a more stable molecular packing than those previously reported[5] and are not readily accommodated by the existing nomenclature which mainly refers to the chain packing. Numerical designations will be used in this article but other terms which have been used are given in the tables.

The controlled slow addition or removal of water from single crystals of phase 2 gave oriented samples of phases 4 and 5. Diffraction experiments showed that the general molecular orientations were retained and lattices corresponding with the different regions of the bilayers were observed, as detailed in Table I.

TABLE II. Data for other DPPC phases.

Phase no.	H_2O %	Preparation method	Transition	Powder data d Å	Notes
2a	10	Crystal solvent excess water	X[a]	3.914 4.367	
6	5	Heat phase 2 to 63.2°C	66.5°C	4.21 57.46 4.07 4.93 14.36	Stability, months at room temp. plastic; $L\beta$
7	5	Heat phase 6 to 66.5°C	67.5°C	X	More stable with less water
8	2–5	Heat phase 7 to 67.5°C crystalline	175°C	4.5 54 4.8	Small thermal peak at 104°C due to water $L8$
9	2.5	Heat phase 8 to 175°C Smectic C	200°C dec.	X	Form observed by DTA and microscopy supercools to 135°
10	>15	Add water to phases 2 or 5	35°C	4.2 64	Hexagonal chain subcell; gel; $L\beta'$
11	>15	Heat phase 10 to 35°C	41.5°C	4.27 66 140	Other long spacings observed; gel; $P\beta'$
12	>25	Heat 11 to 41.5° or add water above 41.5°C	X	4.6 (broad) 54–25% H_2O 61–40% H_2O	Chains disordered "Liquid crystal" $L\alpha$

[a] X, parameters not available.

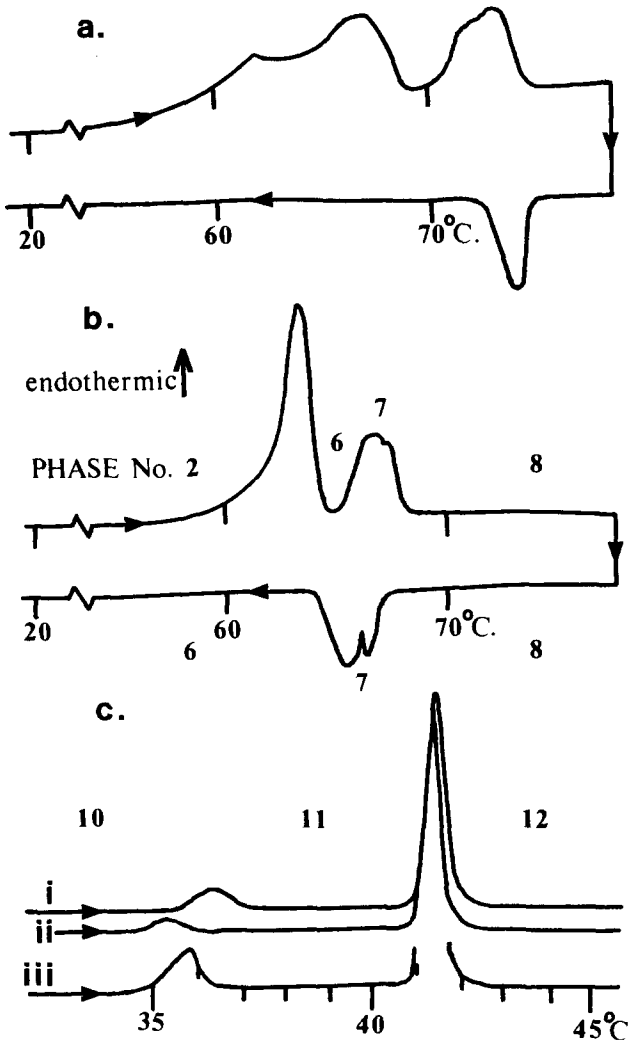

FIG. 1. Differential thermal analysis of 99.94% DPPC, Mettler 2000; sensitivity range, 50 μV a and b, 100 μV c, sealed sample holders. Phase numbers in b and c show stability ranges. Scan rate 0.5°C/min. (a) DPPC phase 2 crystals with trace solvent. (b) DPPC phase 2 crystals, solvent removed without water loss. (c) DPPC with 49% water; (i) first heating; (ii) same sample, second heating; and (iii) after 4 days at 0°C.

The single crystal data given in Table I and other detailed diffraction data were obtained by film and diffractometer, all observations being consistent. Loss of water from crystals of phases 2 and 3 can result in very slight variations in the parameters. Methods used for powder diffraction were described in Ref. 6; small variations may be significant and accurate measurement is desirable. The figures for bilayer repeat distances are in good agreement with values in the literature, but less pure samples show shifts in phase boundaries and broad transitions. Use of thermal analysis with slow scans gives valuable indications of contamination or degradation.[4]

To prepare phase 4, a single crystal was mounted in a chamber with facilities for x-ray diffraction, on evacuation, transitions occurred giving first a rectangular and then a square lattice. A partial transition was observed at about 40 °C which was complete at 50 °C. This region was also explored by thermal analysis of samples dehydrated for different times and by the microscopy of crystals. The changes in diffraction patterns are complex and will be discussed in detail elsewhere.

Phase 5 was obtained by placing crystals in an atmosphere saturated with water vapor for periods of from a few days to one month. Although the edges and corners of the crystals became rounded, a high degree of order was retained and the lattices of phases 2 and 5 could be observed together in several samples.

These experiments showed that special procedures are required to remove solvent traces from crystals of phase 2 without loss of water. The effect of this solvent on thermal analysis is shown in Fig. 1(a) which can be compared with the scan for purified, solvent free, phase 2 crystals in Fig. 1(b). A small difference in the phases formed is also shown by x-ray diffraction. The thermal scans of the purified sample are highly reproducible and reveal that several transitions occur on heating, as detailed in Table II.

These observations indicate that a stable phase 2 and a metastable phase 6 exist for DPPC $2H_2O$, contrary to the conclusions, stated[5] that: "all the phases studied were thought to be in equilibrium." As they have the same composition, the contrast between the relatively hard and brittle crystals of phase 2 and the plastic phase 6 is striking. After storage of phase 6 for several months, recrystallization occurs. The possible formation of stable and metastable phases in the same region of the phase diagram probably accounts for some deviations in the experimental results.

The thermal scans shown in Fig. 1, together with x-ray diffraction, enable the phases produced on heating to be defined. Although transitions also occur on adding water, the location of these phase boundaries has been ill-defined. This problem has been clarified by an analysis based on the details of the molecular packing. This also relates the crystalline phases with those formed in excess water.

As we do not have complete data for highly purified DPPC, results obtained by a variety of authors[7-11] are used which will result in small deviations due to impurity. In Fig. 2, the repeat spacing d is plotted against water content, for a large number of samples. The water content is given as r, the molecular ratio of water/lipid. The points for $r<6$ are for samples at 20 °C but the d spacings change only slightly on heating in this region and there are no transitions below 40 °C.

The data for $r>7$ is for 19 °C or phase 10 and for 37 and 39 °C or phases 10 and 11. Within experimental variations these lie on the same lines. The spread may be partly due to error in water content and hysteresis in the region where a separate water phase appears.

When the molecular volume of the lipid bilayer is constant, addition of water will give an increase in d spacing with a constant slope in Fig. 2 when the area per molecule is constant. When the d spacing remains constant as r increases, an expansion of the area per molecule is indicated. The points for the single crystal

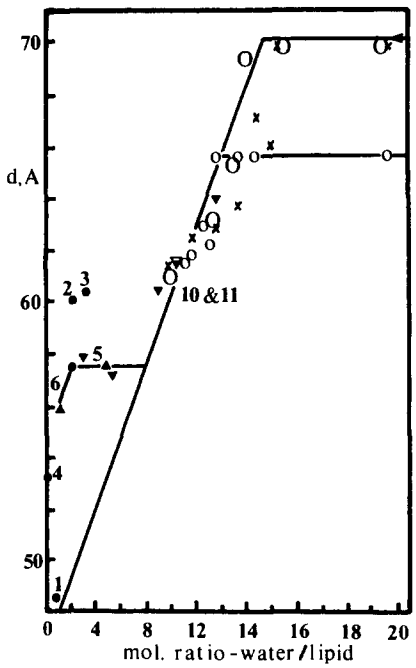

FIG. 2. Variation of bilayer spacing d with water content for DPPC and DMPC. The sloping lines have constant areas of 40.8 Å^2 for phase 6 and 48 Å^2 for phases 10 and 11. Key; ● — phases 1 to 6 listed in Tables I and II, 20°C. ▲ — Ref. 11; ▼ Ref. 7; ▼ Ref. 7, phase 10, all at 20°C. ▽— Ref. 7; DMPC, d+4.4 A, 19°C. o —Ref. 8, 39°C. 0—Ref. 8, DMPC, d+4.4 A, 20°C. ×—Ref. 10, 37°C. → —Ref. 9. This figure is also discussed in Sec. III of this paper.

phases 2 and 3, which are very precise, show increases in both area and spacing, these two phases having virtually the same structure.

The effect of variation in molecular volume and other details of Fig. 2 will be discussed in part III. From Fig. 2, the location of the phase boundaries for the series of phases with hexagonal chain packing is determined.

For phase 6, as the water content increases from $r=1$ to $r=2$, the bilayer spacing becomes longer with the slope corresponding to a constant area per molecule. When $r=0$, there is an increase in area in phase 4.

As r increases above 2, d remains constant up to $r \simeq 7$, so that the area per molecule is increasing and this region is designated phase 5 as being distinct from phases with constant areas per molecule.

When the water content is above $r=8$, the d spacings increase and the experimental data, which is from several sources, corresponds with a constant area per molecule within experimental error. While d again becomes constant at higher r values, this represents the appearance of a separate water phase; so that, for water contents with r greater than 7 there is a phase with constant area per molecule. These discontinuities enable the phase boundaries to be located and, although more detail is required for other regions, greatly improved phase diagrams can be drawn. A diagram showing the phases formed on cooling without precautions to establish the most stable packings is shown in Fig. 3. This resembles previous diagrams. In Fig. 4 are included all well-defined phases presently observed.

On placing crystals of phase 2 in water at 20 °C, they rapidly disperse and transform to phase 10. This undergoes transitions to phases 11 and 12 on heating, as shown in Fig. 1(c). These are the "gel" and "liquid" crystal phases which have been studied extensively as resembling natural membranes. Although the major features are the same, the details in Fig. 1(c) depend on the procedure; the observations are reproducible and will be discussed later. While the crystals rapidly disperse in liquid water, as previously stated, they only take up water very slowly from an atmosphere saturated with water vapor and the quantity of water adsorbed is limited. This difference may be attributed to surface effects which result in distortion of the bilayers in the crystal that minimize the contacts between liquid water and the hydrocarbon chains.

All the phases studied consisted of molecular bilayers in which there is a diglyceride region with only van der Waals intermolecular forces and a polar region with electrostatic and possibly hydrogen bonding forces in addition. The diglyceride region resembles DPG phases which have been studied separately and have many features common with DPPC while the complicating effects of water are absent. For this discussion, the properties of the diglyceride and phosphorylcholine regions will be described first and related to DPPC phases. A division into diglyceride and phosphorylcholine regions permits a sharp physical division between the two

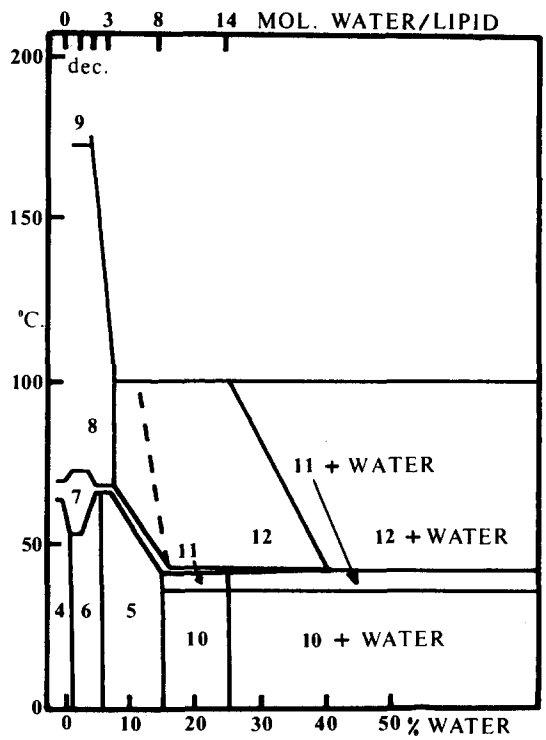

FIG. 3. Phase diagram for high purity DPPC obtained from second heating cycle showing metastable phases.

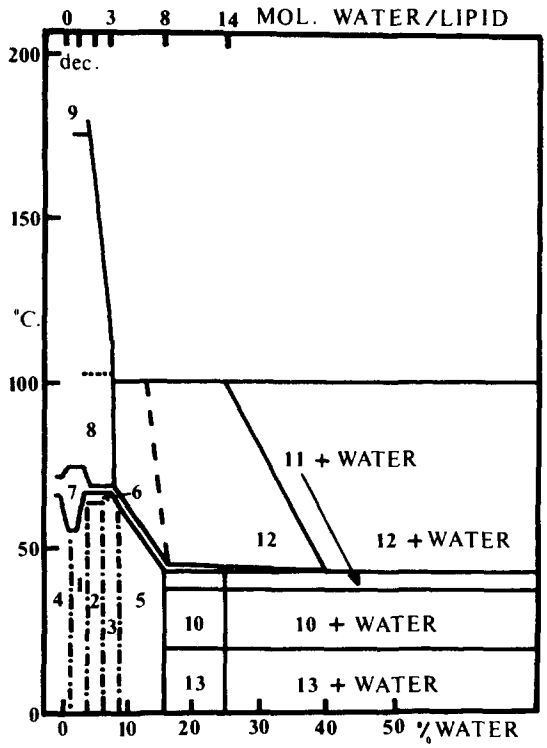

FIG. 4. Phase diagram for high purity DPPC obtained from first heating cycle of phase 2 and from variation in water content. Boundaries obtained also from Fig. 2. The single crystal phases prepared by solvent crystallization are separated by dash–dot lines as they are not known to transform directly into each other. Phase 2 transforms into phase 6 on heating and phases 4, 5, and 10 on change of water content.

regions which does not apply to the common separation into chain and polar regions. This is because the ester groups are flexible while carboxy–ester groups are not.

PROPERTIES OF BILAYER REGIONS

Diglyceride structures; in previous discussions of bilayers, a division into chain and polar regions has usually been made which neglects the significant differences between glycerides and hydrocarbons and a close similarity between several DPG and DPPC phases. The differences are evidently due to the influence of the carboxy–ester groups on molecular packing. While the glycerol molecule is flexible, carboxy–ester groups are planar, the maximum distance of any of the C–C: O–O–C atoms from a plane being 0.08 Å in tricaprin.[12] When two carboxy–ester groups are attached to the same glycerol group by adjacent carbon atoms, the number of stable conformations is restricted.

In DPG phases and the diglyceride region of phospholipids, the molecules are folded with their chain axes parallel. One of the carboxy–ester groups is in the same plane as chain 1 and the other is extended from carbon atom 2 in a plane parallel to the bilayer.[13,14] The properties of all the DPPC and DPG phases studied were consistent with the DPG group retaining this same general shape unless the chains are disordered, as in phase 12.

A detailed study of highly purified DPG[6] revealed the existence of four solid phases with different chain packings. Normal hydrocarbons pack into regular subcells with all the atoms located on a few planes, giving extremely strong x-ray reflections. Several subcells of similar stability are known, with the chains in a regular all-*trans* zigzag to which the subcells are related.

Some hydrocarbons form a phase with hexagonal chain packing over a restricted temperature range near their melting points.[15,16] This subcell is less stable than most other subcells, having longer interchain distances and larger volume. The symmetry has been ascribed to the rotation of an all-*trans* chain but confirmation of this model is lacking and other alternatives are possible. The hexagonal subcell is formed more readily by DPG and DPPC than by hydrocarbons.

Highly purified DPG forms large single crystals with the chains packed into the stable $0_{\perp}$ subcell.[17] This is undistorted compared with the same subcell in hydrocarbon crystals as discussed by Albon and Craievich.[18] The carbon atoms form a planar zigzag with an all-*trans* conformation, although minor deviations are common towards chain ends.

A translation of 2.5 Å along the chain axis direction gives a tilt of about 30° without significant alteration of the subcell as shown by Table III. The chain tilt has a small effect on the molecular volume and decreases the bilayer repeat distance, with an increase in area per molecule in the bilayer plane. For the tilted and nontilted phases of $C_{36}H_{74}$,[19,20] the volume difference is 9 Å^3 or 0.25% per molecule due to changes in end-group packing.[18] The absence of a nontilted phase with $0_{\perp}$ subcell for DPG can be attributed to the lack of space

TABLE III. Molecular volumes and areas of DPPC and PPG phases.

(A) DPG, hexagonal phase, area from d_{100} spacing (Ref. 8)

Temp. (°C)	Area/ (mol Å^2)	Volume/ (mol Å^3)
20	39.31	947
30	39.40	948
40	39.76	952
49	40.49	966
melts		

(B) DPPC at 20°C, from single crystal data

Phase No.	% Water	Volume/	Area/	Volume water	Volume DPPC
1	2.45	1165.5	48.1	28.6	1135.5
2	4.90	1178.7	39.3	28.9	1118.7
3	7.35	1208.5	40.1	29.6	1118.5

(C) Areas per mol. of other DPPC phases

Phase No.	Temp. (°C)	Area/ (mol Å^2)	Calculated from
4	20	41.55	chain subcell
4	20	43.03	polar subcell
6	20	40.74	chain subcell
11	37	42.10	chain subcell

therein for the packing of the carboxy–ester groups. Intermediate angles of tilt are not observed because of the regular interdigitation of the hydrogen atoms along the chain.

Each chain in the $O_\perp$ subcell has six neighbors, two being at a distance of 4.90 Å and the other four at 4.2 Å. An expansion in one direction gives an hexagonal array but the crystalline DPG phase does not transform directly to the hexagonal phase.

On cooling the melt, DPG forms an hexagonal phase, designated $L\beta$ by Craievich *et al.*[6] In this phase each chain is surrounded by six others, all at a distance of 4.85 Å; i.e., they pack as though of cylindrical shape. The greater stability of this phase in DPG and other glycerides than for hydrocarbons is probably due to the carboxy–ester groups. These will influence the spacing between chains and the carboxy oxygen atoms may be located between the cylinders. They also lead to a molecular shape with one dimension twice the other which will slightly favor stacking in rows.

Although details of hexagonal subcell structures are not well established, the subsequent discussion of DPPC structures does not rely on any specific model of the hexagonal phase. The location of the chain axes in the hexagonal subcell is given by the strong diffraction at $d = 4.2$ Å and phases with this packing are smectic liquid crystals when binding between layers is weak, but can form crystals.[21]

Below 24 °C, a small reversible chain tilt occurs in DPG phase $L\beta'$, the change in tilt angle being continuous.[6] This shows that the chains behave as smooth cylinders which require only small forces to slide over each other.

At temperatures below 15 °C, another DPG phase $L\gamma$, is slowly nucleated from $L\beta'$.[6,18] This is more stable than $L\beta'$ and $L\beta$ and the chains are in the all-*trans* conformation. On heating, there is an endothermic transition to the crystalline phase.

Chain packing in DPPC phases; an extensive set of single crystal diffraction photographs is available for DPPC phase 1, from which the chain tilt can be established. To satisfy the unit cell size, symmetry, and molecular dimensions, alternate tilt directions in each layer are required. This involves a packing with crossed chain axes as in potassium caprate.[22] The phase 1 cell data is given in Table I(A), note the twofold axis.

The chains are in the all-*trans* conformation in the crystalline phases 2 and 3, and slightly tilted. In DPPC phases 6, 5, and 10 there is an hexagonal subcell ($L\beta$ and $L\beta'$) which is slightly expanded in phases 4 and 11. In phase 6 the chains are perpendicular to the layers, while in phase 5 the angle of tilt is variable and in phase 10 there is a large constant tilt. In phase 8 ($L8$), there is a square subcell with the same interchain distances as for the hexagonal subcell. Finally, in phase 12 ($L\alpha$) the chains are disordered but a bilayer structure persists.

The relative subcell stability can be estimated from the interchain distances and is confirmed by thermal measurements to be given elsewhere. There is a large decrease from the $O_\perp$ subcell with four interchain distances of 4.2 Å and two of 4.96 Å, through DPPC phase 2 with two of 4.39 Å and four of 5.0 Å, to the hexagonal subcell, with six at 4.85 Å. Although DPPC phases 2 and 6 have similar molecular volumes, the former is more stable because of the number of closer CH_2–CH_2 interactions.

Phosphorylcholine structures; molecular packing in the polar layer will be determined by the charges located on the phosphate oxygen atoms and the choline group and by the size and shape of these groups. These packings are found to be very similar to those in ionic crystals. In these, pairs of ions give square three-dimensional lattices, a face centered lattice being most common but body centered lattices are observed when the ions are of comparable size. The relations between cell dimensions and ionic radii are well established.[1] In general, each charged group is surrounded by others of opposite sign. When one of the charged groups is elongated rather than spherical, the square packing is distorted to give a rectangular cell.

The size and shape of the phosphorylcholine groups can be readily calculated using accepted values of atomic dimensions.[1] The phosphorus and nitrogen atoms are bonded to nearly tetrahedral arrays of oxygen and carbon atoms, respectively. As the carbon to hydrogen bonds in the methyl groups are almost tangential, a spherical shape results for the positive group, with four recesses between the methyl groups. By adding the nitrogen to carbon bond length of 1.47 Å to the van der Waals radius of 1.7 Å, a radius of 3.17 Å is obtained for the choline group. The recess between the methyl groups is too small for another methyl group to penetrate, giving a distance of 6.34 Å for the closest packing of choline groups. This neglects the size of the hydrogen atom, which is more variable, and in choline one hydrogen atom is replaced by a methylene group but these do not alter the minimum packing distance.

The van der Waals radius for phosphorus is 1.9 Å and the phosphorus to (non-ester) oxygen bond lengths are 1.50 Å, with an O–P–O′ angle close to 120°. This gives a distance of 2.60 Å between these oxygen atoms. As the radius of the oxygen atom is 1.4 Å, the negatively charged group has a minimum diameter of 2.8 Å and a maximum of 5.4 Å. These dimensions are from the crystal structures of polar groups.[23,24]

When oxygen atoms are packed with a choline group, they can partly fit into the four spaces between the methyl groups surrounding the nitrogen, as in glyceryl-phosphorylcholine GPC crystals.[24] The shortest nitrogen to oxygen distance observed is 3.81 Å, so that the oxygen atom penetrates by 0.76 Å within the 6.34 Å diameter sphere. This close packing will be sensitive to the motion of the atoms involved.

van der Waals radii vary slightly with the intermolecular forces, the calculated figures given being minimum values. The variation in DPPC phases follows

the usual trends. In the subsequent discussion, a radius of 3.28 Å is used for the choline group, derived from the square polar subcell observed for phase 4 (Table I). This is in excellent agreement with the previous discussion, as also are the values observed for the other groups.

DPPC phases differ from simple ionic crystals in several ways. A significant feature is that the positive ion is larger, usually the reverse is true and this is probably related to the biological functions of the choline phospholipids.

Another difference is that the phosphate and choline groups are joined by a flexible ester link. In compounds with the N–C–C–O group, the molecules fold, placing the nitrogen and oxygen atoms close together. The phosphorus to nitrogen distances in DPPC phases can be assumed to be 4.2–4.5 Å, as in dimyristoyl lecithin,[14] DMPC, and GPC.[24] The distance between the centers of the charged groups will be slightly greater. This places the phosphorus atom close to the choline sphere, with the extra methylene group and the choline ester oxygen atom filling one recess between the methyl groups. The location of the phosphate group relative to the orientation of the nitrogen is variable by rotations of the ester bonds.

Instead of the three dimensional packing found in ionic crystals, the diglyceride group holds the charged groups in layers. Comparison of the length of the phosphate ester group with the combined radii of the choline and carboxy–ester groups shows that the phosphate group cannot be further away from the plane of the carboxy–ester group 2 than the center of the choline group. The structures also depend on the forces in the diglyceride layer and the water content.

INFLUENCE OF WATER ON DPPC PHASES

The effects of water on DPPC phases are striking and complex. Chapman *et al.*[25] reported that anhydrous DPPC adsorbs water from P_2O_5 and that larger amounts of water are weakly bound. In this work, highly purified DPPC crystallized as the monohydrate from solvents in contact with dry molecular sieve. This water is removed only by heating above 200 °C at atmospheric pressure, which also results in some decomposition of the DPPC molecule. The chemical formulas usually given for lecithins include a water molecule. Anhydrous DPPC can be obtained by prolonged storage in vacuum.

In crystals of the polar groups, hydrogen bonds are formed with the non-ester oxygen atoms and this involves a water molecule joining two phosphate groups in the cadmium chloride complex of GPC.[26] A network of hydrogen bonds was also reported for the structure of DMPC dihydrate.[14] The geometry of hydrogen bonding to polar groups has been established by structural studies,[13,14,23,24,26] and would restrict the packing of the charged groups.

While the phospho-diester group tends to form hydrogen bonds, the quaternary nitrogen group is strongly alkaline and binds water as the hydroxyl ion. The water molecules will still be held between the phosphate

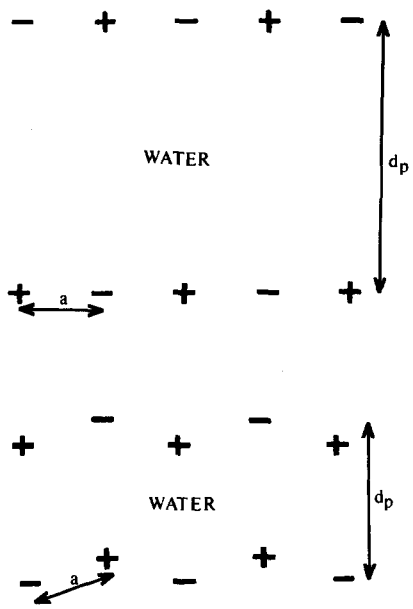

FIG. 5. Diagram showing effect of interaction between layers of charged groups (top) excess water, very weak forces (bottom), at low water content a more stable array is formed by movement of the choline groups.

oxygen atoms and the choline groups by electrostatic forces but will not restrict the geometry as much as when they form hydrogen bonds. Any intramolecular binding of water is prevented by the shape of the phosphorylcholine group.

Analysis of molecular packing in the polar regions of DPPC phases suggested that the influence of the choline group was predominant. This has recently been confirmed by the Raman spectroscopy of purified samples which will be described separately.[27] The formation of stable mono, di, and trihydrate phases by DPPC can be ascribed to the preferential binding of three water molecules to the choline group. These will be partially located between the methyl groups and will be closer to the nitrogen atom than other water molecules.

As the water content is further increased, it is held between the layers of charged groups and forms an hydration layer similar to the hydration spheres around charged molecules in solution. Although the area per molecule increases, the polar groups in each layer do not become separated by water unless their chain length is very short.

When excess water is present, as in phases 10, 11, and 12, the polar groups of different bilayers do not interact appreciably and in each layer the charged groups will pack in contact in a planar array as illustrated in Fig. 5, top. The forces between polar groups across each bilayer will tend to compress the bilayer. This contributes to the reduction in bilayer thickness above the main transition, in phase 12, when the forces between the chains are weak.

For water contents below $r = 7$, as in phases 6 and 4, the electrostatic forces between the charged groups of different bilayers increase. The choline groups, or

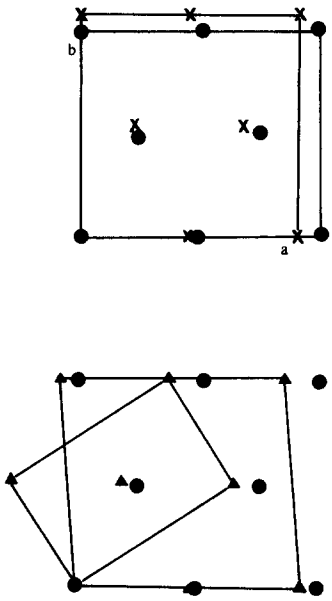

FIG. 6. Top: chain packing in DPPC phase 2, × and in phase 6, ● . Both are dihydrates and the packing in 6 is hexagonal. Bottom; chain packing in DPG phase $L\gamma$ ▲ and in DPG phase $L\beta$ ● . The latter is hexagonal and identical with DPPC phase 6. An orthorhombic cell is shown for the $L\gamma$ phase with axes of 7.58 and 5.02 Å. The structures are viewed along the chain axes.

water molecules attached to them, can more easily move towards the other bilayer as shown in Fig. 5, bottom. If the distance between the charged groups in each bilayer remains the same, the area per molecule in the bilayer plane will be reduced and the lipid bilayer spacing will increase, after allowing for the water layer. The movement reduces the forces between the polar layers, both across and between bilayers. There is an equilibrium position at which the repulsive and attractive forces on the moving charges balance, this equilibrium will vary with the distance between the charges, that is on the water content, as in phase 5. The equilibrium will also be influenced by the orientation of the water molecules, changes in dissolved molecules, and differences in potential across the bilayer.

This discussion leads to polar region structures having coplanar choline groups and phosphate oxygen atoms for phases 10, 11, and 12 in excess water. The shape of the negatively charged group gives rectangular arrays of molecules. In phases with γ less than 3, the choline groups will be closely packed with the phosphate oxygen atoms displaced towards the bilayer center planes. A detailed description based on the parameters given here will be presented in parts II and III.

Molecular volumes and areas: The relative stability of DPPC phases is determined by the packing in each region. For comparisons in the diglyceride region between phases with the chain axes parallel, the area per molecule can be used although interchain or interatomic distances are more precise. Areas per molecule in the bilayer plane can be used to establish molecular packing in the polar region when unit cells are not available. A large amount of precise data has been obtained which gives information about the changes in volumes and areas which occur in DPG and DPPC phases both on heating and during transitions.

The expansions of DPG phases on heating were accurately measured and are highly directional.[6] From these results,[6] the variation with temperature of the molecular volume and area of the hexagonal DPG $L\beta$ phase were calculated and are given in Table III(A).

The volumes and areas for the DPPC single crystal phases are given in Table III(B), these are much more accurate than previous estimates and will be referred to later. In Table III(C) are the areas per molecule for several other DPPC phases, the apparent discrepancy for phase 4 being due to a small chain tilt.

Comparison of DPG and DPPC phases; DPG and DPPC phases are similar except for the absence of a tightly packed subcell in DPPC phases. An area of 37.17 $Å^2$ for the DPG $0_\perp$ subcell in the crystalline phase compares with 39.29 $Å^2$ for DPPC phase 2 and 40.49 $Å^2$ for DPG phase $L\beta$ near its melting point; which is very close to the values for hydrocarbons near their melting points and for DPPC phase 6 at 20 °C. Vand[16] gives 20.30 A^2 per chain for n-hexatriacontane at 75 °C.

Because of the chain tilt, the area per molecule in the bilayer plane for the DPG crystalline phase is 41.50 A^2, which is nearly the same as for DPPC phase 6 [Table III(C)]. However, the polar groups evidently cannot be packed in a manner compatible with the cell dimensions of the DPG crystalline phase.

The DPG $L\beta$ phase appears to be identical in structure with the diglyceride region of DPPC phase 6, with the chain axes perpendicular to the bilayer plane, the former melts at 49 °C to a liquid, while the latter transform at 66.5 °C to another solid phase. The structure of this will be discussed in part II, but clearly, the polar packing stabilizes an ordered phase at an higher temperature.

DPG phase $L\beta'$ is very similar to DPPC phases 5 and 10, with an hexagonal chain packing having tilted chain axes. A variable angle of tilt is observed as a result, of temperature changes in one phase, and from the variation of water content in the other.

Finally, the chain packing in DPG phase $L\gamma$ is very similar to that in DPPC phases 2 and 3, and in both these phases the chains are in a planar zigzag. The relations between chain lattices in these phases are shown in Figs. 6 and 7.

For both DPG and DPPC, the phases with hexagonal chain packing form readily on cooling a melt or another higher temperature solid phase. In contrast, DPG phase $L\gamma$ is difficult to nucleate and crystallizes slowly from the solid, although more stable than the hexagonal phases. DPPC phase 2 shows a similar behavior. These stable phases are associated with a difference in chain packing and a similar stable phase has now been observed in aqueous dispersions of DPPC.[28] The prediction can be made that the chain packing in this phase will resemble that in DPG phase $L\gamma$ and DPPC phases 2 and 3. This phase has been numbered 13 in

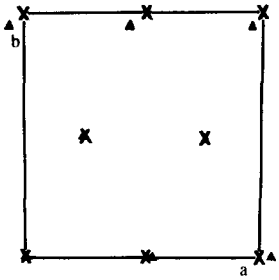

FIG. 7. Comparison of chain packing in DPPC phase 2, × with that in DPG phase $L\gamma$,▲.The packing in DPG $L\gamma$ is intermediate between that in DPPC phases 2 and 6 and the chain form planar zigzags as in phase 2.

the phase diagram shown in Fig. 4.

The relation of the crystalline, stable DPPC monohydrate phase 1 to the other phases, differs from that of phases 2 and 3. In this structure there is a very stable polar group packing associated with weak binding in the chain region, as shown by the relatively large molecular volume [Table III(B)].

Comparing the melting temperatures of DPG phases with the appearance of chain disorder in DPPC–water systems, the most striking feature is the absence of disorder below 200 °C for $r = 2$ with DPPC. For DPPC in excess water the disorder appears at a temperature which is 7.5 °C lower than the melting point of the DPG, $L\beta$ phase, so that the influence of the polar group, when in contact with water, is smaller.

Nature of transitions in lipid systems; there has been considerable speculation about the nature of the phase transitions in lipid systems and the study of highly purified samples, with well defined structures, provides greatly improved data.

These results show that melting of the DPG crystalline and $L\beta$ phases are isothermal first order transitions similar to those for other pure crystalline substances. A previous report[4] confirmed conclusively that this is also true of the appearance of chain disorder at the "main" transition in DPPC aqueous dispersions (phase 12). The conclusions to be presented in part III are that the polar regions are only disordered in phase 12 in that there is increased libration above the transition. However, there was no evidence of order–disorder transitions occurring over appreciable temperature ranges in purified DPPC.

The reversible chain tilt observed in DPG phase $L\beta'$ and in DPPC phase 5 may be classed as second order transitions. On this basis, phase 5 represents a finite transition region between phases 6 and 10.

Many of the other transitions between two ordered phases appear to have finite widths, but these are small. The thermal scans often are asymmetric in shape.

The existence of many phases indicates that there are several ways of packing the molecules with similar stability to which both regions of the bilayer contribute. As the intermolecular forces in these regions differ, changes in temperature or water content will alter the balance between them. A notable feature of DPPC–water systems is that the packing in the diglyceride region does not attain the maximum effectiveness. While the ester group joining the regions allows some difference in their packing, each molecule must occupy the same area in the bilayer plane.

The ordered structures in the diglyceride regions can be classified as those with chains in the regular planar zigzag as for the DPPC crystalline phases 1, 2, and 3. Another class has chains which seem to occupy smooth cylinders, with hexagonal packing in DPPC phases 4, 6, 5, 10, and 11 and a square packing in DPPC phase 8. Formation of structures permitting greater atomic motion (although still ordered) as in the hexagonal packing usually occurs on heating. In DPG and DPPC phases, lattice expansion due to the carboxy–ester and polar group packings has a similar effect.

Structures in the polar region are closely related to those in ionic crystals. When the water content is low, the choline groups pack in contact in a plane displaced from that of the phosphate oxygen atoms. At higher water content, these groups form a closely packed coplanar array which is evidently so stable that it is also formed when the monohydrate is crystallized from solvents. On heating, the chains are disordered in phase 12, this is shown to correspond to an increased libration of the polar groups but not to involve disorder of these groups.

The molecular packings in DPPC phases with r less and more than 7 will be discussed in Sec. II and III of this article, respectively, this division corresponds to regions having the bilayers in contact and separated by water. In all phases the packings are closely related and follow the same rules as for all other compounds.

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