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PHASE TRANSITION PROPERTIES OF AQUEOUS DISPERSIONS OF HOMOLOGUES OF ALL-*TRANS* 2,3-DIPALMITOYLCYCLOPENTANO-1-PHOSPHOCHOLINE

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In a previous publication, (Singer, M.A., Jain, M.K., Sable, H.Z., Pownall, H.H., Mantulin, W.W., Lister, M.D. and Hancock, A.J. (1983) Biochim. Biophys. Acta, 731, 373–377), we reported the properties of aqueous dispersions of the six diastereo-isomers of cyclopentanoid analogues of dipalmitoylphosphatidylcholine. Two of these isomers displayed unusually high enthalpies of transition, about double that of dipalmitoylphosphatidylcholine. One of the high enthalpy isomers whose configuration is all-*trans* has now been modified by the insertion of extra methylene residues ($n = 3$ through 9) between the nitrogen and phosphorus atoms of the headgroup. Vesicles were formed from these lipids and studied by ^{22}Na permeability measurements, differential scanning calorimetry, fluorescence polarization, ^{31}P-NMR, and freeze-fracture electron microscopy. Vesicles composed of lipids with $n = 2$ or 3 exhibit a sharp transition at 46°C or 49°C, respectively, and a high enthalpy with no detectable sub- or pretransitions. Lipids with $n > 3$ exhibit a main transition between 38 and 43°C with enthalpies < 10 kcal/mol and after prolonged cooling (more than 3 days at 4°C) a broad endotherm at about 20 ± 3°C with enthalpies > 4 kcal/mol. These same dispersions display a permeability peak at 20–25°C and a second increase in ^{22}Na efflux in the temperature range 30–40°C. The results of ^{31}P-NMR measurements suggest that the acyl chains in 2,3-dipalmitoylcyclopentano-1-phosphocholine ($n = 2$) bilayers have restricted rotation below the main phase transition temperature.

Introduction

The conformational changes accompanying various thermotropic phase transitions of synthetic phospholipids in bilayers are becoming better understood [1]. One of the approaches to elucidate the contributions of various structural features of a phospholipid molecule is to study the phase transition properties of conformationally and orientationally restricted analogues [2–6]. Thus by systematic covalent linking and modification of the different regions of phospholipid monomers one can modulate the orientation and motional freedom of the molecules in the bilayer. The phase properties, which depend upon the packing arrangement of the phospholipids, will be modified accordingly.

Recently, we reported the phase transition properties of the six diastereo-isomers of cyclopentanoid analogues of dipalmitoyl phosphatidylcholine [6]. While each of these phospholipids formed liposomes which exhibited a sharp thermo-

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tropic transition, two of the isomers exhibited unusually high enthalpies of transition. In order to investigate the origin of this high enthalpy, we have modified one of these isomers by varying the number of methylene residues between the phosphate and ammonium groups (Fig. 1). The properties of these synthetic phospholipids suggest that this modification of the headgroup modulates the motional freedom of these molecules in the bilayer and thereby influences the various thermotropic transitions. The high enthalpy of the parent isomer appears to be due to a direct transition of the acyl chains from a close-packed crystalline-like packing to the liquid-crystal state.

Materials and Methods

2,3-Dipalmitoylcyclopentano-1-phosphocholines ($n = 2$ through 9) (Fig. 1) were synthesized as described elsewhere [7]. All the studies reported here were done with procedures already established in our laboratories. Differential scanning calorimetry (DSC) was performed with a Mettler 2000 B calorimeter [8]. Lipid dispersions were prepared in distilled water and between 2 and 4 mg lipid was added to each DSC pan. Scan rates were 1 $K \cdot min^{-1}$. Fluorescence polarization measurements were done on an SLM 4800 spectrofluorimeter in T-configuration [4] and ^{31}P-NMR was done on a Bruker WM 250 [4].

Vesicles to be used for efflux measurements were prepared by drying down an appropriate aliquot of a stock chloroform solution of a given lipid and dispersing the dried lipid in 50 mM NaCl/5 mM Tris-HCl/^{22}NaCl (3 μCi/ml), pH 7.5, above the lipid transition temperature. The vesicles were dialyzed overnight at 5 °C against buffer lacking the isotope to remove untrapped tracer and then incubated at different temperatures in stoppered glass tubes to measure the efflux rates [9].

Freeze-fracture microscopy was done with an Edwards freeze-fracturing and etching unit. Vesicles were prepared in distilled water containing glycerol 25% (v/v). The samples were maintained at 22 °C until the time of freezing. Grids were examined in a Hitachi model Hs-9 electron microscope.

The structure of the cyclopentanoid analogues

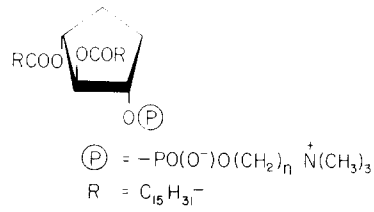

Fig. 1. Structure of the cyclopentanoid analogues. The parent compound has $n = 2$ and an all-*trans* configuration. It was designated as 1,3/2-1P in Ref. 6. The homologues used in this study have $n = 2$ through 9.

of phosphatidylcholine is given in Fig. 1. For the sake of simplicity, the compounds will be referred to by the number (n) of methylene groups in the headgroup where $n = 2$ is the parent compound (1,3/2-1P of Ref. 6) and $n = 3, \ldots, 9$ are the new homologues with elongated headgroups.

Results

Aqueous dispersions of $n = 2$ through 9 give stable liposome suspensions capable of trapping ions. Fig. 2 illustrates the changes in ^{22}Na efflux as a function of temperature, for vesicles formed from these lipids. For $n = 4$ through 9, there is a large increase in permeability in the temperature region 30–40 °C and a smaller but significant permeability peak at 20 °C ($n = 5$, 7, 9) or 25 °C ($n = 4$, 6, 8). For $n = 2$, the ^{22}Na efflux rate remains high at all temperatures examined and there are no obvious temperature dependent permeability peaks. This curve for $n = 2$ is similar to the one reported previously in Ref. 6. Finally, for $n = 3$, the ^{22}Na permeability profile is similar to that of $n = 2$ with, however, a more obvious increase over the temperature range 20 °C to 35 °C. These changes in permeability are most likely due to changes in the phase properties of the bilayers. As shown below, the differential scanning calorimetry of these liposomes shows a main transition in the region of 38 to 49 °C and for those vesicles composed of lipids with $n > 3$ a subtransition in the temperature region of 20 °C. However, as discussed later, the correspondence between permeability changes and DSC transitions in the 15 to 30 °C range in not yet clear.

The main thermotropic transition seen in the ion leakage and DSC experiments was also observed in the temperature dependence of the

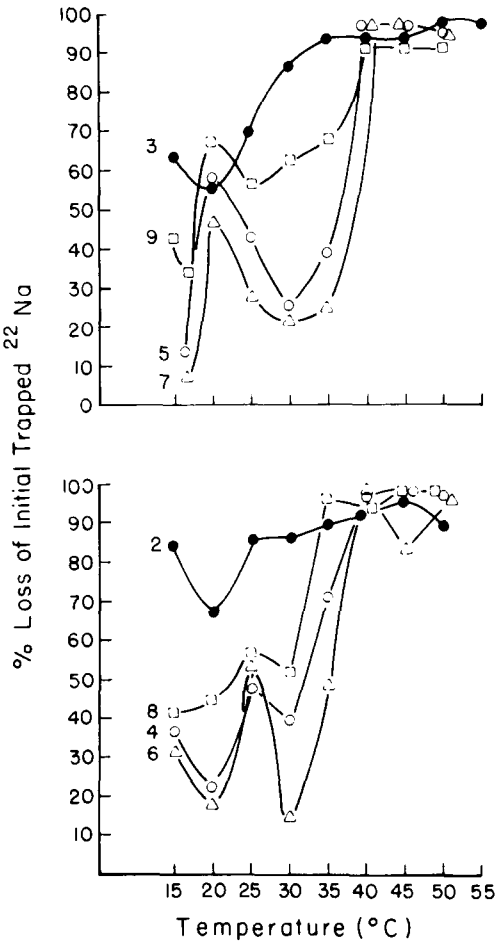

Fig. 2. ^{22}Na effluxes in different vesicles (n = 2–9) at different temperatures. Vesicles composed of the lipids indicated in the figure were swollen in 50 mM NaCl/5 mM Tris-HCl/^{22}NaCl (pH 7.5). The ordinate refers to the percentage of initial trapped ^{22}Na lost over 180 min (3 h). Experimental points for each temperature represent one, or in some cases, two separate experiments each performed in triplicate. The results illustrated are the mean values. To simplify the figure, error bars have not been given but the range of values did not exceed 25% for any of the mean values.

steady-state fluorescence anisotropy (r) of polarization of diphenylhexatriene. The anisotropy for the aqueous dispersions of the various lipids studied in this paper (data not shown) was 0.34 ± 0.01 below their main transition temperature, and 0.08 ± 0.03 above their transition *. These values

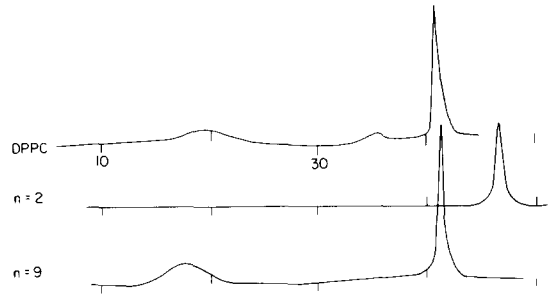

Fig. 3. DSC profiles for dipalmitoylphosphatidylcholine (DPPC) and the cyclopentanoid analogues. These scans were done on aqueous dispersions which had been stored for 3–4 days at 4 °C, using a Mettler 2000 B instrument at 100 μV sensitivity and a scan rate of 1 K/min between 1 and 80 °C. Each sample contained 2–4 mg of the lipid dispersed in distilled water. The scans for n = 4 to 8 are similar to that of n = 9 except for the position of the main transition which occurred within a temperature range of 38–43 °C (see Fig. 4). The subtransition for n = 4–8 had a similar temperature position to that of n = 9. DPPC had a T_m of 41.9 °C and an enthalpy of 8.7 kcal/mol.

are close to those seen for dispersions of dipalmitoylphosphatidylcholine under similar conditions. We did not observe any reproducible temperature-dependent change in anisotropy of polarization that could be ascribed to the subtransitions measured by DSC for n > 3.

Recently the thermotropic transition behavior of phospholipid dispersions has suggested the existence of several metastable states below the main transition [10–16]. Typically, dipalmitoylphosphatidylcholine dispersions stored for three or more days at 4 °C exhibit three endothermic transitions which have been labelled as the sub-, pre-, and main transitions. A DSC scan for such a dipalmitoylphosphatidylcholine liposome preparation is illustrated in Fig. 3. As also shown in Fig. 3, n = 2 dispersions, treated in the same fashion (i.e. stored for more than three days at 4 °C), exhibit only a single endothermic transition at 46 °C with an enthalpy value of 19 kcal/mol. The transition temperature, the enthalpy, and the shape of the transition profile remain unchanged on repeated scans. The high enthalpy is also observed on repeated heating as well as in the cooling cycle, although the phase transition temperature is lowered to 38 °C in the cooling cycle **.

* The phase transition temperatures obtained by these measurements were typically about 0.5 to 2 K lower than those observed by DSC.

** In DSC scans we did not observe any pretransition for any of the homologues n = 2 through 9.

The thermal properties of dispersions where $n = 2$ are consistent with the hypothesis that the main transition for this phospholipid is actually a composite of all three transitions observed in dipalmitoylphosphatidylcholine liposomes. The subtransition phase of dipalmitoylphosphatidylcholine corresponds to a bilayer 'crystal' state in which the acyl chains interact closely in a 'quasi-orthorhombic' arrangement. If this hypothesis is valid we reasoned that by increasing the size of the phosphocholine headgroup it should be possible to push the acyl chains apart and thereby isolate the individual phase states of these chains. Indeed as shown in Fig. 3, analogues in which $n > 3$ exhibit multiple thermal transitions. Typically, these lipids exhibit a sharp endothermic transition in the 38 to 43 °C range, which is also seen in the cooling cycle and on the second heating cycle. A broad endothermic transition at 20 ± 3 °C is also observed in the first heating cycle. This transitions at the lower temperature is not seen in the cooling cycle or in the second heating cycle. This lower temperature transition reappears only when the samples are stored at 4 °C for several days. Based on this behavior we believe that this lower temperature endotherm corresponds to the subtransition observed in dipalmitoylphosphatidylcholine dispersions. It shoud be emphasized that the enthalpy of the subtransition is strongly dependent upon the history of the sample. Some samples incubated for long periods (several months) exhibit a very large endotherm (> 15 kcal/mol) in the subtransition and in some cases even the main transition is somewhat modified. For the compound $n = 2$, we have always seen a single transition at 46 °C in the first heating run even after storage at 4 °C for six weeks.

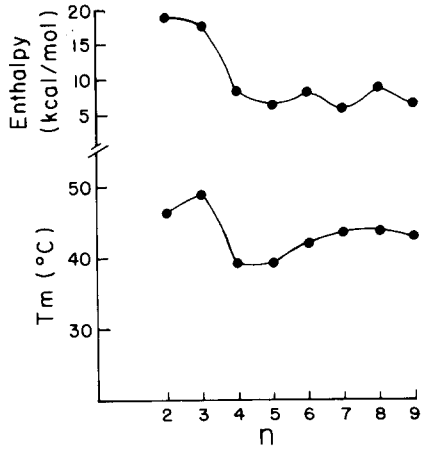

Fig. 4. Main transition temperatures (T_m) and enthalpies of cyclopentanoid analogues. The abscissa refers to the number (n) of methylene groups between the nitrogen and phosphate in the headgroup. T_m refers to the peak of the main transition (± 0.1 K). Enthalpy values are given in units of kcal/mol with an error of $\pm 10\%$ in the extreme. The T_m and enthalpies were derived from the first DSC scan obtained immediately after cooling the given lipid dispersion preheated above its main transition, (that is, the second scan).

For the homologue, $n = 3$, the thermal transition profile is very complex. Several endo- and exothermic transitions are detectable in the first heating cycle whose temperature and enthalpy depends upon the scanning rate and the storage history of the sample and were not always reproducible even for the same sample.

Fig. 4 summarizes the main transition temperatures and enthalpies for the series $n = 2$ through 9 obtained from the second heating runs. For $n = 2$ or 3 the transition enthalpy is quite high, about 19 kcal/mol. For $n > 3$ the enthalpy falls to a value less than 10 kcal/mol and remains relatively constant thereafter. The transition temperature itself appears to display a minimum value at $n = 4$ or 5. In addition there is no odd/even effect on the transition temperature as has been described for a comparable series of analogues of dipalmitoylphosphatidylcholine in which additional methylenes have been inserted into the headgroup [17].

In order to gain some insight into the molecular motion of these lipids when dispersed into vescles, we examined their ^{31}P-NMR line shapes which depend both on the orientation of ^{31}P-tensors and on the rotational freedom of these molecules in the bilayer [4]. As shown in Fig. 5, the ^{31}P-NMR line shape for $n = 2$ liposomes at 30 °C is broad compared to that observed for dipalmitoylphosphatidylcholine under the same conditions [10]. The 2,3-dipalmitoylcyclopentano-1-phosphocholine dispersion thus shows a 'slow motion' spectrum, below the main phase transition, indicating an incomplete motional averaging of the nonaxially symmetrical ^{31}P-shift tensor [4,18]. The ^{31}P-NMR line shape for dipalmitoylphosphatidylcholine dispersions above 15 °C [10] and for $n = 2$ above 45 °C approach an axially symmetrical pattern indicating a near-complete motional averaging due

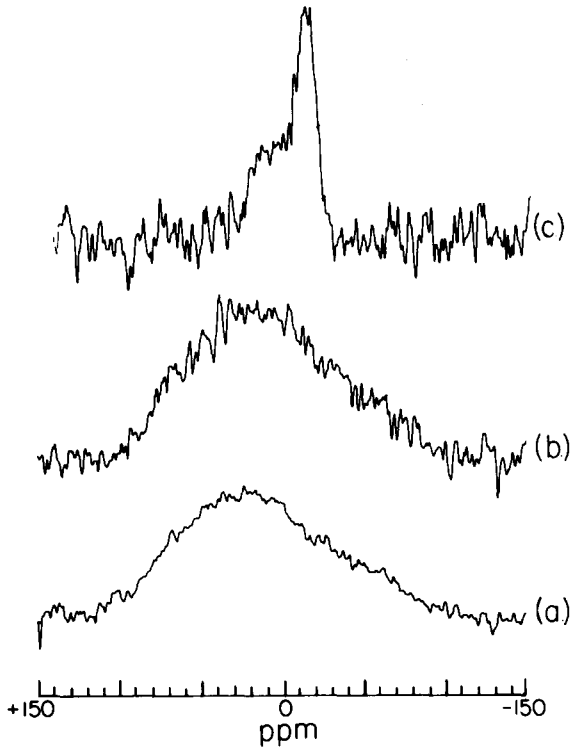

Fig. 5. ^{31}P-NMR spectra of $n = 2$ dispersions. These spectra were obtained at (a) 8 °C, (b) 30 °C, (c) 50 °C. As described by Fuldner [10], spectra run below 12 °C for dipalmitoylphosphatidylcholine dispersions after storage at 4 °C for sufficient time for a subtransition to appear, are similar to those of $n = 2$ run at 8 °C and 30 °C but above 12 °C dipalmitoylphosphatidylcholine dispersions given a spectrum similar to that in (c).

to rotational freedom. However, a slow-motion spectrum for dipalmitoylphosphatidylcholine dispersions is seen in the crystalline phase [10], formed by incubation of the liposomes for several days at 4 °C. These observations demonstrate that the organization of $n = 2$ in aqueous dispersions below 45 °C is similar to that in the aqueous dispersions of dipalmitoylphosphatidylcholine below their subtransition temperature [10–12]; that is, they have restricted rotation presumably due to close packing of the chains.

Finally, Fig. 6 illustrates the morphology by freeze fracture microscopy of several of these analogues as well as dipalmitoylphosphatidylcholine for comparison. Vesicles were freshly prepared and then quenched from 22 °C. Dipalmitoylphosphatidylcholine and $n = 4$ display smooth surfaces characteristic of the gel phase [19] while the surface of vesicles of $n = 2$ is quite different and has a crinkled texture.

Discussion

It is now apparent that the thermal properties of phospholipids at temperastures below the main phase transition are quite complex. This behavior has been best studied with respect to the phosphatidylcholines. Storage of these lipids at a low temperature (at about 4 °C) for variable periods of time leads to the appearance of several transitions below the main gel to liquid-crystal phase change [10–13]. These subtransitions are a consequence of interconversion between metastable states. The rate of formation of these states is dependent, among other factors, upon acyl chain length [13]. Although the exact packing arrangement in these states has not been clearly defined it is known that the acyl chains are closely approximated and that the rotational freedom of the lipid molecules is severely restricted. The observations reported in this paper suggest that bilayers composed of $n = 2$ or $n = 3$ below their main transition temperature contain lipid molecules organized in a close-packed state analogous to the 'crystalline' packing displayed by dipalmitoylphosphatidylcholine dispersions in one of its metastable states. On the basis of this hypothesis the high enthalpy observed for the main transition of $n = 2$ or $n = 3$ dispersions can be ascribed to a direct change from a phase similar to the 'orthorhombic' crystalline packing to a liquid-crystal state.

The total enthalpy of $n = 2$ dispersions is approximately equal to the sum of enthalpies of the sub- and main transitions of 1,3-dipalmitoylphosphatidylcholine (β-DPPC) [20] and it is somewhat higher than the sum of enthalpies for 1,2-dipalmitoylphosphatidylcholine (α-DPPC) [11–13]. The difference between α- and β-DPPC is ascribed to differences in the acyl chain packing and the orientation of the headgroup in the bilayer. Low angle X-ray diffraction studies of 2,3-dipalmitoylcyclopentano-1-phosphocholine dispersions are in progress. However, electron diffraction studies on the epitaxial crystals of these lipids suggest that there are 'striking' dissimilarities between the acyl

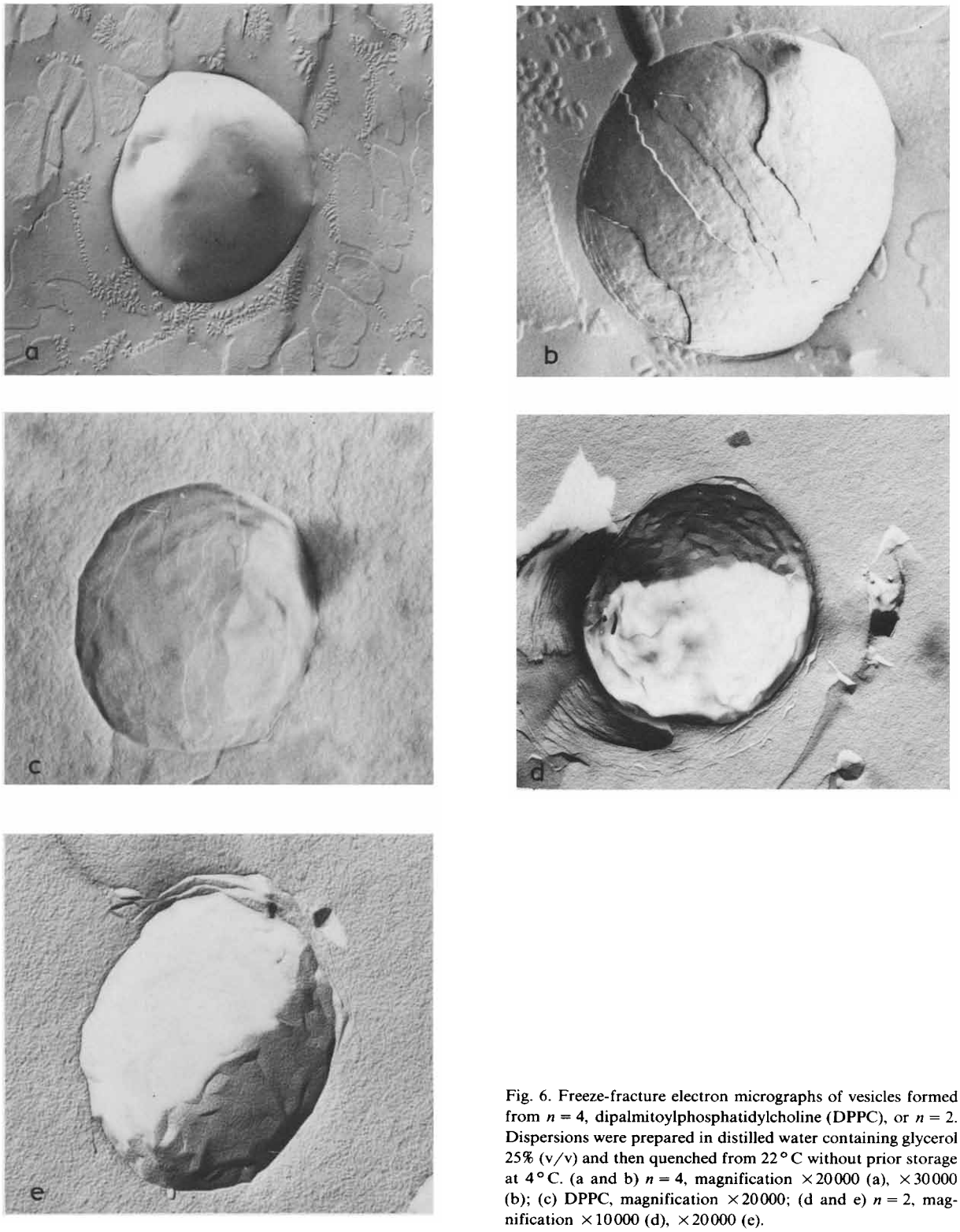

Fig. 6. Freeze-fracture electron micrographs of vesicles formed from $n = 4$, dipalmitoylphosphatidylcholine (DPPC), or $n = 2$. Dispersions were prepared in distilled water containing glycerol 25% (v/v) and then quenched from 22 °C without prior storage at 4 °C. (a and b) $n = 4$, magnification $\times 20000$ (a), $\times 30000$ (b); (c) DPPC, magnification $\times 20000$; (d and e) $n = 2$, magnification $\times 10000$ (d), $\times 20000$ (e).

chain packing of $n = 2$ dispersions and α-DPPC at 30 °C [21].

The thermotropic behavior of $n = 2$ or 3 bilayers is dramatically altered by increasing the phosphate to nitrogen distance by the insertion of extra methylene residues. Dispersions formed from cyclopentano lipids with $n > 3$ develop a subtransition when stored at 4 °C for several days, indicating that for these lipids the formation of metastable states is a much slower process than for $n = 2$ or 3. The larger headgroups appears to retard close acyl chain packing under ordinary conditions.

It is of interest that the vesicles formed from cyclopentanolipids $n = 4$–9, display a permeability change not only in the temperature vicinity of 30 to 40 °C but also in the region of 20 to 25 °C. The former is probably a result of conversion of the lipid to the liquid-crystal state as has been well described for glycerol-based diacyl lipids [9]. The permeability 'peak' at 20 to 25 °C could be a consequence of interconversion between metastable states, that is, associated with a subtransition. However, the correlation between changes in permeability properties and DSC transitions below the temperature of the main phase change is still speculative.

Vesicle morphology as visualized by freeze-fracture electron microscopy revealed distinct differences among these lipids. Freshly prepared dispersions of dipalmitoylphosphatidylcholine and $n = 4$, quenched at 22 °C, display smooth surfces characteristic of the gel phase [19]. It is tempting to speculate that the crinkled surface texture of freshly prepared $n = 2$ vesicles also quenched at 22 °C is the morphologic correlate of the close-packed crystalline-like phase.

In summary, the experimental results reported in this paper suggest that the high enthalpy of transition of dispersions formed from $n = 2$ and probably $n = 3$ is due to a direct change from a crystalline close-packing to a liquid-crystal state.

Acknowledgements

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