*Biochimica et Biophysica Acta* 854 (1986) 1–8
Elsevier

BBA 72847



# Organization of chlorophyll *a* in bilayer membranes. The chlorophyll *a* / dimyristoylphosphatidylcholine system

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(Received July 2nd, 1985)

Key words: Phospholipid bilayer; Differential scanning calorimetry; NMR; Chlorophyll-lipid interaction

**In order to investigate the relative importance of the hydrophobic and headgroup interactions of chlorophyll *a* in phospholipid bilayers, we have carried out differential scanning calorimetry (DSC) and deuterium ($^2$H) and phosphorus ($^{31}$P) nuclear magnetic resonance (NMR) experiments on the multilamellar system of chlorophyll *a* in dimyristoylphosphatidylcholine (DMPC). Compared to the phytol chain of chlorophyll and the previously reported distearoylphosphatidylcholine (DSPC), the acyl chains of DMPC are shorter in length by three and four carbons, respectively. A lowering in the phase-transition temperature was observed for the DMPC multilayers in the presence of chlorophyll *a* in the DSC thermograms and in the $^{31}$P chemical shift anisotropy measurements. These results, together with data on hydrophobic interactions as measured by $^2$H-NMR and on headgroup interactions as evidenced from $^{31}$P-NMR, suggest a phase diagram for the chlorophyll *a* / DMPC system in which phase separation readily occurs between a chlorophyll-rich compound phase and a chlorophyll-poor phospholipid phase. Compound formation appears to be important in the stabilization of chlorophyll *a* in bilayers with shorter chains.**

## Introduction

The light-harvesting events of photosynthesis are known to take place in the thylakoid membrane. While the function of chlorophylls in the photosynthetic process is reasonably well defined, the structural organization of the light-harvesting chlorophylls in the thylakoid membrane is a subject of considerable current interest. Several studies have suggested that chlorophyll-protein complexes closely represent the state of the bulk of antenna chlorophyll in vivo [1–3]. An extensive study of the resonance Raman of chlorophyll *a* in chloroplasts suggests, however, that chlorophyll *a* must be bound to a number of different moieties in the chloroplast membrane [4]. $^{13}$C-NMR studies of intact thylakoid membranes [5] have, in fact, demonstrated that some of the galactolipids and chlorophyll phytol chains give rise to high-resolution spectra, suggesting that a pool of chlorophyll molecules is associated with the lipid portion of the thylakoid, or possibly bound at the periphery of membrane proteins.

Several groups [6–10] have introduced chlorophyll *a* into phospholipid bilayer membranes and have demonstrated that the chlorophyll can be integrally incorporated into the bilayer, with the porphyrin headgroup in the polar region of the membrane and the nonpolar phytol group inserted

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** Contribution No. 7212 of the Department of Chemistry, California Institute of Technology.

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

into the hydrophobic core of the membrane along the fatty acyl chains. The phase diagram of chlorophyll *a* in a bilayer membrane of distearoylphosphatidylcholine (DSPC) has been mapped by Eigenberg et al. [9,10], showing that the organization and intermolecular interactions of chlorophyll *a* with DSPC vary widely depending on the temperature and composition of the system. These workers also obtained evidence for compound formation between chlorophyll *a* and DSPC involving interaction of the lipid phosphate with the magnesium ion in chlorophyll.

The work of Eigenberg et al. emphasized the interaction of the lipid phosphorus headgroup with the magnesium ion in chlorophyll. Moreover, they selected a phospholipid where the hydrophobic interactions between the phytol chain and the hydrocarbon chain of the lipid are optimized, i.e., the chain lengths are about equal when the stearic acyl chain is fully extended. To gain some insight into the contributions of the hydrophobic forces in chlorophyll-lipid interactions, we have now carried out differential scanning calorimetry (DSC), and deuterium ($^2$H) and phosphorus ($^{31}$P) nuclear magnetic resonance (NMR) experiments on the multilamellar system of chlorophyll *a* in dimyristoylphosphatidylcholine (DMPC). Compared to DSPC and the phytol chain of chlorophyll, the acyl chains of DMPC are shorter in length by approximately four carbons. A comparison of the phase behavior of chlorophyll *a* in DSPC and DMPC should permit a delineation of the relative importance of the hydrophobic and headgroup interactions in the stabilization of chlorophyll *a* in the bilayer.

## Materials and Methods

Chlorophyll *a* was isolated from spinach extracts following the procedures of dioxane precipitation [11] and powdered-sugar chromatographic purification [12,13]. Final purification was carried out in milligram quantities with high-pressure liquid chromatography, using an Alltech 5 $\mu$m $C_{18}$ reverse-phase column. Complete resolution of chlorophyll *a* was achieved using a mobile phase consisting of 6% water in methanol at a column flow rate of 4 ml/min. Care was taken to minimize exposure of the chlorophyll *a* samples to room light and to keep the samples under nitrogen.

DMPC was purchased from Sigma Chemical Company. Chain perdeuterated dimyristoylphosphatidylcholine ($d_{54}$) was from Avanti Polar Lipids, Inc., Birmingham, AL. The purity of the lipids was checked prior to use by silica gel thin-layer chromatography, using a mobile phase consisting of chloroform/acetone/acetic anhydride/methanol/water (50 : 20 : 10 : 10 : 5, v/v). Deuterium oxide and deuterium-depleted water were obtained from Aldrich Chemicals.

Multilayers were made by dissolving the lipid or a mixture of lipids in chloroform in an NMR tube, and then evaporating the solvent under a stream of nitrogen. The residual solvent was removed by drying the sample under vacuum overnight. The dried film was then hydrated with a 25 mM Tris buffer solution (p$^2$H 7.4) by alternately vortexing on a mixer and gentle warming to just above the phospholipid gel-to-liquid-crystalline phase-transition temperature. The vortexing was continued for about 5 min until a uniform, white suspension was formed. The samples all contained a 1 : 10 w/v ratio of lipid to water.

Small vesicles were prepared by transferring the multilayer suspension to a centrifuge tube and sonicating with a Heat Systems-Ultrasonics, Inc. model W-225R cell disruptor as previously described [10]. The solution was then centrifuged to remove particles eroded from the sonicator tip and also small amounts of large bilayer structures. Negative-stain electron microscopy showed that small vesicles about 50 nm in diameter were obtained using this procedure. NMR measurements were carried out on vesicle solutions prepared just prior to their use.

Samples for differential scanning calorimetry (DSC) were prepared by transferring an appropriate amount of the chlorophyll *a*/DMPC multilayers to a preconditioned, large-volume stainless-steel sample container and hermetically sealed with a Perkin-Elmer Quick Press. The reference pan contained an equivalent volume of distilled water.

Differential thermal analysis was carried out on a Perkin-Elmer DSC-4 Differential Scanning Calorimeter with a microprocessor temperature-programmer and a thermal-analysis data station.

Both sample and reference were heated at a uniform rate of 5 Cdeg/min from 8 to 60°C.

NMR spectra were acquired at 11.7 T (202.49 MHz $^{31}$P and 76.78 MHz $^{2}$H frequency) on a Bruker WM 500 spectrometer equipped with an Aspect 2000 computing system, and a variable-temperature unit which maintains the temperature of the sample constant to ±0.5 Cdeg. $^{31}$P-NMR spectra were obtained using a broadband probe under gated broad-band proton decoupling at a power of 10 W using a spectral width of 50 kHz, a 75° pulse and a relaxation delay of 1.5 s. Deuterium NMR spectra were obtained using a high-power deuterium-proton double-tuned probe with an rf 90° pulse of 5.5 μs and using the quadrupolar echo pulse technique [14]. A 166 kHz spectral width and a refocusing time of 50 μs were used.

## Results

*Differential scanning calorimetry*

The effect of increasing chlorophyll *a* concentrations on the differential scanning calorimetry properties of DMPC is shown in Fig. 1. Two thermal transitions were observed for the pure DMPC multilamellar system: a low-enthalpy pretransition with an endotherm maximum at 14°C and a high-enthalpy chain-melting transition with an onset temperature at 24°C, similar to the thermogram reported by Janiak et al. [15]. In the presence of a few percent chlorophyll *a*, the pretransition could no longer be observed; the main transition was broadened and started at a lower temperature. This transition was reversible and was observed on successive heating cycles. With increasing chlorophyll *a* concentration (> 0.15 mole fraction), the transition was further broadened, but no additional lowering in the onset of the gel-to-liquid-crystalline transition temperature was observed. The initial decrease in the onset of the gel-to-liquid-crystalline phase-transition temperature and the broadening of the main endothermic peak reflect changes in the bilayer structure resulting from the interaction of the DMPC with chlorophyll *a*. Only a single peak was observed for the chlorophyll *a*/DMPC system, in contrast to the thermograms of chlorophyll *a* in DSPC which showed two distinct peaks over the concentration range corresponding to chlorophyll *a* mole fractions of 0.08–0.30. The overall thermal phase diagram for the hydrated chlorophyll *a*/DMPC system appears simpler than the chlorophyll *a*/DSPC system.

*$^{31}$P-NMR*

We have studied the effect of chlorophyll *a* on the $^{31}$P-NMR spectrum of the DMPC multilamellar system. The characteristic features of the $^{31}$P-NMR spectrum of phospholipid multilayers have been described by several research groups [16,17]. A plot of the DMPC $^{31}$P chemical shift anisotropy ($\Delta\sigma$) as a function of temperature at several chlorophyll *a* concentrations is shown in Fig. 2. A lowering in the gel-to-liquid-crystalline phase-transition temperature was observed, consistent with the DSC data, indicating that the chain melting phenomenon in DMPC multilayers was significantly perturbed by the presence of chlorophyll *a*. Some typical spectra at selected composition at

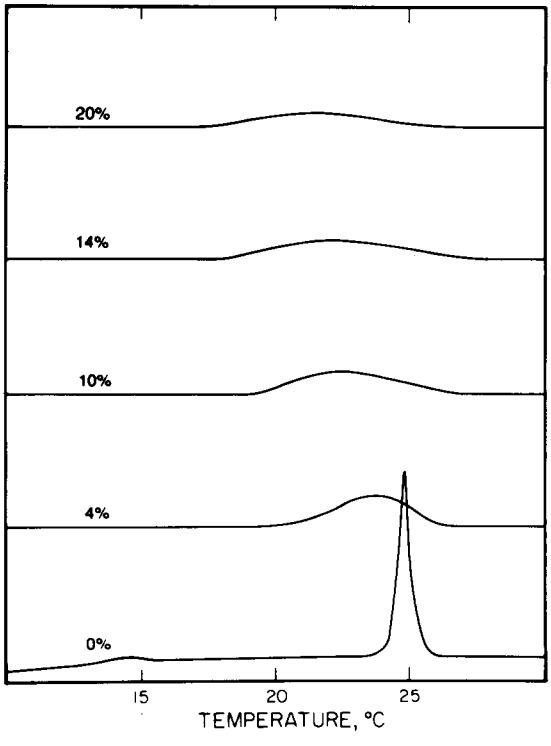


Fig. 1. The effect of chlorophyll *a* on the DSC thermogram of hydrated DMPC multilayers in excess water.

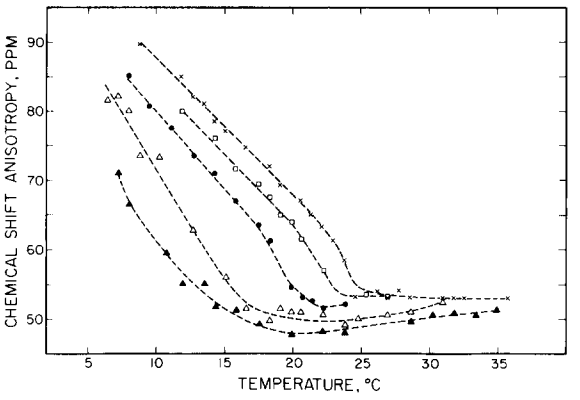


Fig. 2. Graph of $\Delta\sigma$ versus temperature showing the effect of increasing chlorophyll *a* concentration on the $^{31}P$ chemical shift anisotropy of the lipid phosphorus in DMPC multilayers. ×, pure hydrated DMPC multilayers; DMPC multilayers containing: □, 0.04; ●, 0.10; △, 0.14; ▲, 0.32 mole fractions of chlorophyll *a*.

26°C are shown in Fig. 3. Although the presence of chlorophyll *a* had little effect on the chemical shift anisotropy above the phase-transition temperature, there were differences in the line-shapes of the $^{31}P$-NMR spectra when the chlorophyll *a* mole fraction exceeded 0.10. The linewidth of the upfield peak and the intensity of the low-field peak were observed to increase with increasing chlorophyll *a* concentrations, indicating that another component centered around 1 ppm might be present. In addition, the area of the central component was also found to be temperature-dependent; the $^{31}P$-NMR spectrum of a 0.32 mole fraction chlorophyll *a* sample at 30°C is compared with that at 20°C in Fig. 4.

Computer simulation of the $^{31}P$ multilamellar spectra showed that the central component observed in the chlorophyll *a*/DMPC system could be caused by the presence of multilayers with a smaller chemical shift anisotropy around 30 ppm. At 26°C, this component was estimated to be present to the extent of 4% and 10%, respectively, in the DMPC samples containing 0.14 and 0.32 mole fractions of chlorophyll *a*. Thus, the line-shape observed in the chlorophyll *a*/DMPC multilamellar system could be caused by a phase separation between two phases of well-defined composition, such as compound and solution.

This possibility prompted us to examine the

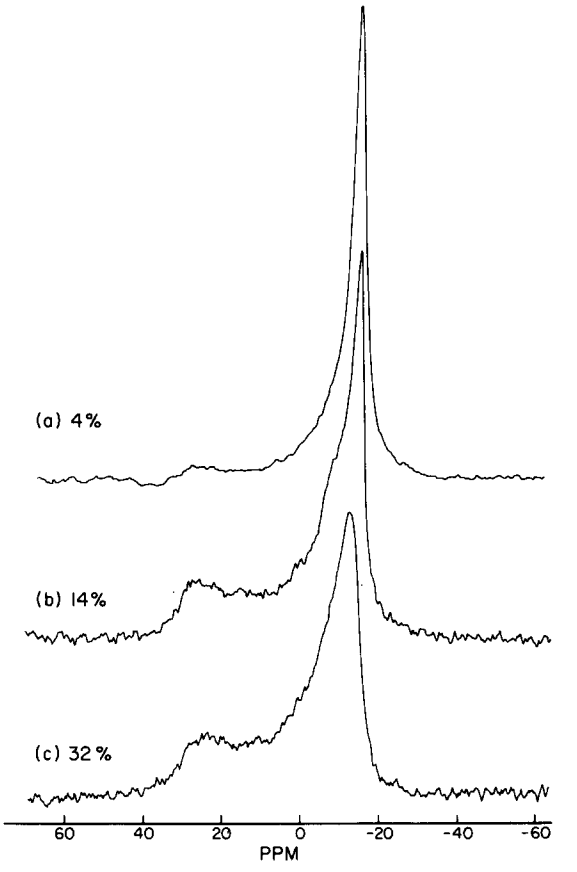


Fig. 3. $^{31}P$-NMR spectra of lipid headgroup phosphorus in chlorophyll *a*/DMPC multilayers at 26°C: (a) 0.04, (b) 0.14 and (c) 0.32 mole fractions of chlorophyll *a*.

$^{31}P$-NMR spectra of the chlorophyll *a*/DMPC vesicle system. Fig. 5a, b and c depicts the 202.49 MHz $^{31}P$-NMR spectra of a 0.20 mole fraction chlorophyll *a*/DMPC vesicle suspension at three selected temperatures. Below 46°C, two phospholipid resonances were observed, the relative area ratio of which was temperature-dependent. A single peak was observed at temperatures above 46°C. The total area of the phosphorus resonance was approximately the same at high and low temperatures, indicating that all of the lipid was portioned into essentially only two slow-exchange environments at the lower temperatures, where phase separation apparently occurred.

The corresponding $^{31}P$ spectra for a chlorophyll *a*/DMPC vesicle suspension containing 0.32 mole fraction of chlorophyll *a* are shown in Fig. 5d, e

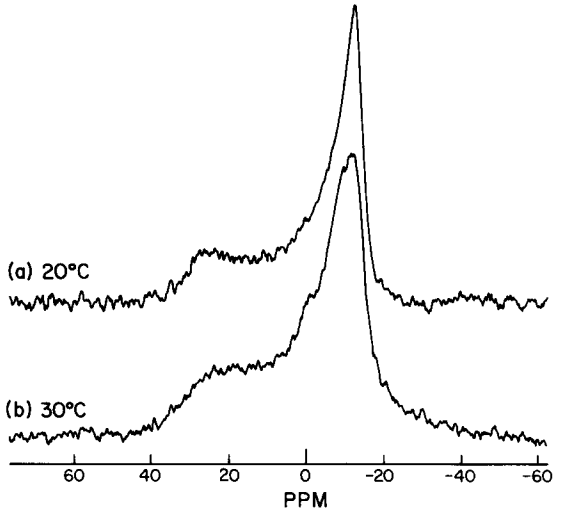


Fig. 4. $^{31}$P-NMR spectra of lipid headgroup phosphorus in 0.32 mole fraction chlorophyll *a*/DMPC multilayers at (a) 20°C and (b) 30°C.

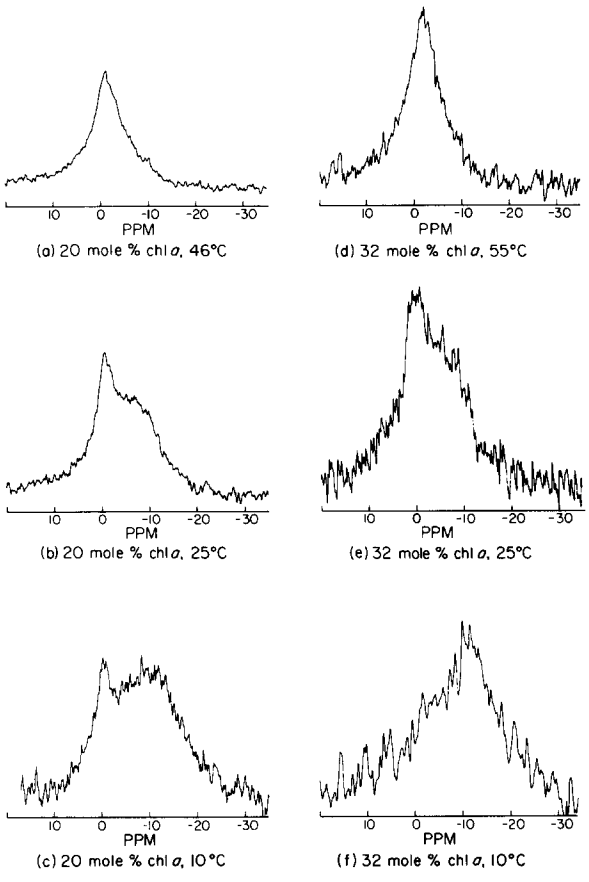


Fig. 5. 202.49 MHz proton-decoupled $^{31}$P-NMR spectra of: 0.20 mole fraction chlorophyll *a*/DMPC vesicles at (a) 46°C, (b) 25°C and (c) 10°C; 0.32 mole fraction of chlorophyll *a*/DMPC vesicles at (d) 55°C, (e) 25°C and (f) 10°C.

and f. For this sample, two phospholipid resonances were observed below 55°C, with chemical shifts essentially identical to those observed for the 0.20 mole fraction chlorophyll *a*/DMPC vesicles. A homogeneous lipid mixture was obtained only at temperatures above 55°C. The phosphorus peak area at 55°C was approximately the same as the sum of the two peaks observed at lower temperatures. One of the phospholipid peaks discerned at the lower temperatures had a chemical shift nearly the same as that of the single peak observed in the high-temperature spectrum; the other peak was shifted 5.8 ppm upfield. This additional upfield resonance has been noted previously in the chlorophyll *a*/DSPC system [10] and has been assigned to the phospholipid headgroups in the compound phase, where coordination between the lipid phosphate moiety and the central magnesium atom of the chlorophyll has been postulated.

### *$^2$H-NMR*

The $^2$H-NMR spectrum of a 10% (w/v) multilamellar dispersion of perdeuterated dimyristoylphosphatidylcholine (DMPC-$d_{54}$) in deuterium-depleted water at 25°C is compared with that of DMPC-$d_{54}$ containing 0.15 mole fraction of chlorophyll *a* in Fig. 6. These spectra represent a superposition of the powder patterns arising from all the chain positions labelled. The higher intensities in the outer spectral extremes were associated with a plateau of high order for the first several segments of the acyl chain [18,19]. Deuterium quadrupolar splittings as measured here are directly proportional to the order parameters of the labelled segments, and as such are indicators of the flexibility of the hydrocarbon chains. Although the overall shapes of the DMPC-$d_{54}$ spectra with and without chlorophyll *a* are similar, it is evident that the positions of the peaks were different in the presence of chlorophyll *a*. The magnitudes of the quadrupolar splittings for the deuterons along the chain in DMPC-$d_{54}$ and in the presence of chlorophyll *a* are compared in Table I. While not all the deuterons were resolved in the perdeuterated chain and the assignments of the chain

TABLE I

DEUTERIUM QUADRUPOLAR SPLITTINGS FOR BILAYERS OF PERDEUTERATED DIMYRISTOYLPHOSPHATIDYLCHOLINE WITH AND WITHOUT CHLOROPHYLL *a*

| Tentative labelled carbon atom | Deuterium quadrupolar splittings (kHz) | | | |
|---|---|---|---|---|
| | DMPC | | DMPC with 15 mole % Chl *a* | |
| | 25°C | 32°C | 25°C | 32°C |
| C-2–9 | 27.8 | 26.0<br>24.8 | 30.9 | 27.1 |
| C-10 | 25.6<br>23.8 | 22.5<br>20.8 | 29.4<br>27.3 | 25.0<br>22.8 |
| C-11 | 23.8<br>21.0 | 20.8<br>18.2 | 27.3<br>23.5 | 22.8<br>19.9 |
| C-12 | 20.0<br>18.0 | 17.2<br>15.5 | 22.7<br>20.1 | 18.5<br>16.5 |
| C-13 | 16.0<br>13.3 | 13.6<br>11.3 | 18.0<br>14.5 | 14.4<br>11.9 |
| C-14 | 3.7 | 3.2 | 4.0 | 3.3 |

deuterons were tentative, an approx. 10% increase in the quadrupolar splittings was observed for the deuterons in all chain positions in the presence of 0.15 mole fraction chlorophyll *a*. No additional significant changes in the quadrupolar splittings were observed as the chlorophyll *a* mole fraction was increased further to 0.32, even though the linewidth of the quadrupolar lineshape became somewhat broader due to heterogeneity arising from increased dispersion of quadrupolar interactions or motional effects. Cooling these chlorophyll *a* lipid samples below 19°C resulted in hydrocarbon chain crystallization into the rigid-crystalline gel phase and a broad, rather featureless spectrum was obtained.

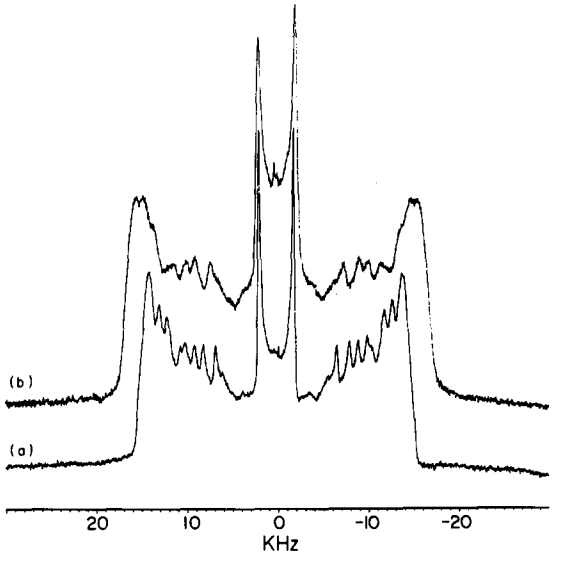


Fig. 6. 76.78 MHz $^2$H-NMR spectra of (a) pure hydrated chain perdeuterated DMPC, and (b) hydrated chain perdeuterated DMPC with 0.15 mole fraction of chlorophyll *a* in deuterium-depleted water at 25°C.

## Discussion

The deuterium NMR results obtained here, which show an increase in the value of the order parameter along the entire length of the acyl chain with chlorophyll *a*, provide the first conclusive evidence that the phytol chain of chlorophyll *a* is deeply inserted into the hydrophobic region of the membrane. The multilamellar structure for DMPC persists even at high chlorophyll *a* concentrations. The linewidths in the $^2$H-NMR spectra are quite broad in the presence of chlorophyll *a*, suggesting that signals with a range of different effective quadrupolar couplings may be present due to a distribution of order parameters among the chains; the slow motions as reflected in the spin-spin quadrupolar chain relaxation may be affected as well.

The DSC and NMR studies presented here suggest a phase diagram for the chlorophyll *a*/DMPC bilayer in which the DMPC phase-transition temperature is lowered in the presence of chlorophyll *a* and that a phase separation between a chlorophyll-rich compound phase and a chlorophyll-poor phospholipid phase readily occurs. The phase state of the system and the intermolecular interactions between DMPC and chlorophyll *a* are dependent on both the temperature and the composition of the system. The transition temperatures for the various compositions are plotted to yield the two-component phase diagram which is shown in Fig. 7, with water, the third component, being present in large excess.

The phase diagram in Fig. 7 is characteristic of compound formation between chlorophyll *a* and the phospholipid. At zero chlorophyll concentra-

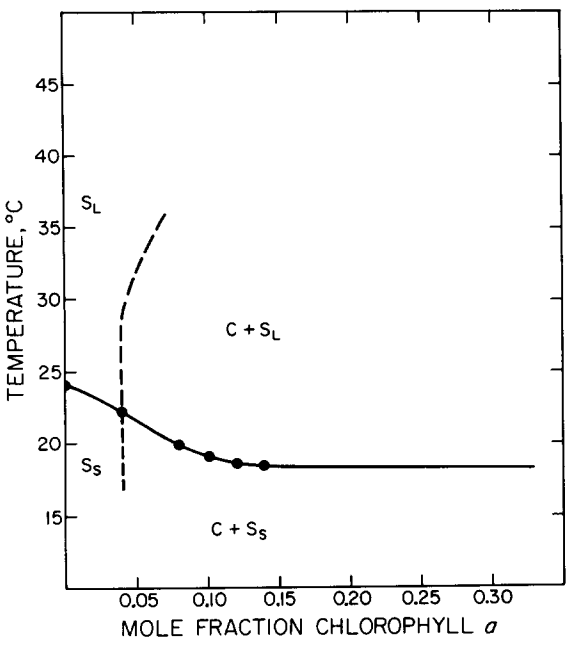


Fig. 7. Phase diagram of the chlorophyll *a*/DMPC multilamellar suspension in excess water. The data points indicate phase-transition temperatures obtained from DSC data. Abbreviations: $S_L$, liquid-crystalline multilayer solution; $S_S$, solid solution or gel phase; C, compound phase.

tion, the phase behavior of pure DMPC has been well characterized. Above the main transition temperature of 24°C, DMPC multilayers are in a fluid-like liquid-crystalline phase ($S_L$). At lower temperatures, DMPC multilayers exist in a more solid-like and ordered gel-phase ($S_S$). Regions $S_L$ and $S_S$ are therefore homogeneous one-phase regions which consist of a fluid-like solution of chlorophyll *a* in DMPC bilayers for the $S_L$ region, and a solid solution of the same for the $S_S$ region. Compound formation between chlorophyll *a* and the phospholipid DMPC occurs when the chlorophyll *a* mole fraction exceeds 0.04. This is evident from both the unusual $^{31}P$ line-shape of the multilayers and the appearance of two $^{31}P$ resonances in the sonicated vesicle system. The observed upfield shifts of 5.8 ppm in the vesicle samples are identical to that previously reported for the chlorophyll *a*/DSPC system, which implies that the lipid phosphate and chlorophyll magnesium lie in a close proximity in the compound phase, probably within 4 Å, in order to account for the large upfield shift.

While the phase diagram for the chlorophyll *a*/DMPC system is reminiscent of the phase diagram for the chlorophyll *a*/DSPC system, the co-existence of the chlorophyll-rich and chlorophyll-poor regions over a large region of the phase diagram indicates that DMPC and chlorophyll *a* are less miscible compared to the DSPC and chlorophyll *a* system. In samples containing more than 4 mole percent chlorophyll *a*, a homogeneous population of DMPC can be found only at relatively high temperatures. This provides an interesting contrast to the chlorophyll *a*/DSPC system, where a homogeneous liquid-crystalline phase was present above the gel-to-liquid-crystalline phase-transition temperature, and phase separation was observed only below the liquidus. Furthermore, the region where both liquid crystalline and gel phases co-exist is not observed in our NMR experiments. Such a region probably occupies only a very small region in the chlorophyll *a*/DMPC phase diagram. Thus, compound formation involving a coordination interaction between the lipid phosphorus headgroup and the central magnesium atom of chlorophyll appears to be important in the stabilization of chlorophyll *a* in bilayers with shorter chains.

## Acknowledgement

This work was supported in part by Grants GM-22432 (S.I.C.), GM-08117 (P.D.) from the National Institutes of General Medical Sciences, U.S. Public Health Service, and the Petroleum Research Fund Grant No. 16046-B4 (P.D.) administered by the American Chemical Society. The use of the Southern California Regional Facility at the California Institute of Technology was supported by the National Science Foundation Grant No. ChE 79-1632. We thank Drs. U. Banerjee and R. Zidovetzki for helpful discussions, and S. Kos of Perkin Elmer for technical assistance with the DSC.

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