MONODOMAIN SAMPLES OF DIPALMITOYL PHOSPHATIDYLCHOLINE WITH VARYING CONCENTRATIONS OF WATER AND OTHER INGREDIENTS

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ABSTRACT Methods are presented for the preparation of large monodomain phospholipid bilayer arrays containing variable amounts of water approaching the two-phase limit. The optical birefringence of these lamellar phases of dipalmitoyl phosphatidylcholine (DPPC) is measured over a range of temperature and water content, and phase transitions are observed. The techniques employed for pure DPPC and water are extended in order to produce macroscopically aligned samples containing varying concentrations of cholesterol, inorganic salts, antibiotics, and chlorophyll *a*. Polarization studies of the 670-nm band of chlorophyll *a* indicate macroscopic orientational order in the chromophore under the same conditions.

INTRODUCTION

Lipid bilayers are generally recognized as useful models of biological membranes. It is also well known that in certain ranges of temperature and concentration, mixtures of lipid and water form homogeneous lamellae, or lyotropic smectic liquid crystalline phases, consisting of lipid bilayers separated by layers of water (1, 2). A number of different techniques for obtaining single-domain smectic samples in which the bilayers are held flat and stacked parallel to one another have been developed in recent years (3–7). The most successful of these techniques in terms of the maximum area (1 cm^2) and thickness ($\sim 10^5$ bilayers) has recently been described for the monohydrates of dipalmitoyl phosphatidylcholine (DPPC) and egg yolk phosphatidylcholine (8). These monohydrate systems produced by Powers and Clark (8) have limited application. We will describe here an extension of their technique by which one can obtain macroscopically oriented samples containing any amount of water up to the two-phase limit. In addition we will describe achievement of macroscopically aligned samples of DPPC containing inorganic ions, cholesterol, both carrier and pore antibiotics, and chlorophyll *a* at varying water concentrations and temperatures.

The macroscopic orientation of these samples were monitored principally by optical microscopy and conoscopy while varying temperature and water content. Conoscopy

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is an excellent technique for detection of phase transitions and one of the results to be reported below is an optically determined phase diagram for DPPC as a function of water and temperature. The effects on the phase diagram due to other ingredients will also be discussed.

To the extent that other membrane-associated molecules can be incorporated into these aligned macroscopic samples without altering their basic biological properties, these samples provide a unique tool for polarized spectroscopic studies of the ingredients as oriented by the lipid lamellae. Results demonstrating the feasibility of studying the polarization dependence of chlorophyll *a* spectra in aligned DPPC multilamellae will be described.

METHODS

Materials

Synthetic DPPC was obtained from Calbiochem (San Diego, Calif.). Thin-layer chromatography was done both on lipid fresh from the bottle and also on lipid extracted from the aligned samples at different water concentrations. Only those samples that indicated a purity in excess of 99.5% were accepted. In addition lipid samples equilibrated in excess water were placed in a capillary and observed with an optical microscope while the capillary temperature was varied. The optical texture of the lipid was observed to change reproducibly from clear when $T > 41.8°C$ to waxy below this temperature. This agrees with published values of the "gel" transition temperature for pure DPPC in excess water (9).

The silane surfactant essential to the alignment procedure of Powers and Clark (8), *N*, *N*-dimethyl-*N*-octadecyl-3-aminopropyltrimethoxysilyl chloride, was obtained from Dow Corning Corp. (Midland, Mich.) (10).

Cholesterol and valinomycin were purchased from Calbiochem and used without further purification. Nonactin was purchased from E. R. Squibb and Sons (Princeton, N.J.) and gramicidin A from ICN Pharmaceuticals, Inc., (Irvine, Calif.). Chlorophyll *a* was extracted from spinach leaves and purified by the method of Strain and Svec (11).

All of these materials were stored in the dark under dry nitrogen and refrigeration ($\sim 0°C$).

Alignment Procedures

PURE DPPC + WATER In all cases to be described here the methods for obtaining macroscopically oriented samples are variations on the annealing procedure described by Powers and Clark (8). Two glass slides are suitably cleaned and treated with the silane surfactant *N*, *N*-dimethyl-*N*-octadecyl-3-aminopropyl-trimethoxysilyl chloride to produce a lipophilic surface that will orient the first DPPC monolayer to come in contact with it (10). In place of the glass slides one can also use conducting metal or metal oxide surfaces as substrates. An alternative to the silane surfactant, hexadecyltrimethylammonium, can be applied to the substrate in the manner described by Kahn et al. (10). However, it appears to be less effective in achieving alignment than the silane surfactant.

In the method of Powers and Clark (8) for pure DPPC, the desired amount of lipid, containing between 2% and 8% water by weight, is placed between two surfactant-treated surfaces separated by a spacer to fix the overall sample thickness. The entire assembly is then clamped together as indicated in the schematic diagram (Fig. 1). If the spacers are made of Teflon or some other material of comparable mechanical properties, the effect of the clamp is to seal the sample. Materials such as glass or Mylar can also be used to make the spacers.

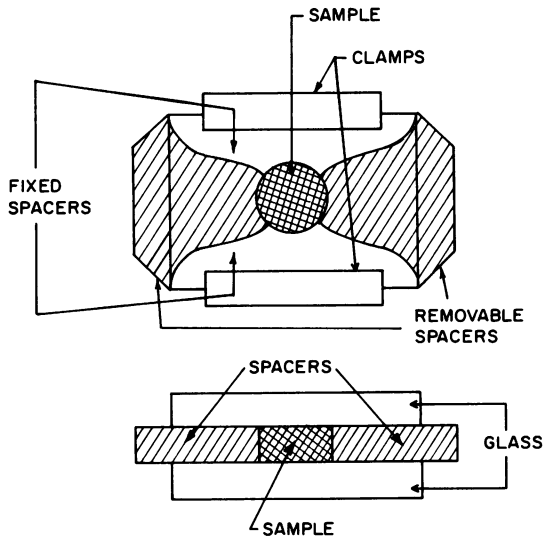

FIGURE 1

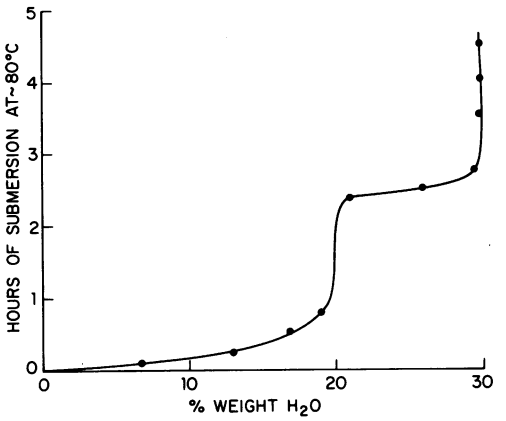

FIGURE 2

FIGURE 1 Schematic diagram of the sample assembly. Top, face view; bottom, side view with one clamp removed. The drawing is not to scale; however, the spacing can vary from 25 μm to 500 μm thick, the sample diameter is approximately 1 cm, and the glass slides are roughly 3 cm × 5 cm.

FIGURE 2 Submersion time (at ~80°C) as a function of added water for oriented samples of DPPC. Oriented samples of monohydrate are submerged at ~80°C and after the indicated time the sample was removed and allowed to equilibrate for 4–6 h. The sample was then brought to room temperature and water content was determined by dry weight. The sample thickness was 125 μm.

The sample assembly described above is heated to approximately 120–125°C and kept there for 4–6 hr. At this temperature and water content, the combination of planar boundary conditions induced by the surfactant and the macroscopic elasticity of the lamellar phase makes it energetically unfavorable to have a multidomain sample. If the sample has reasonably high fluidity, the multidomain character will anneal away, in that smaller domains will merge to form larger ones and the larger ones will grow further until the sample becomes all one single domain. For pure DPPC the only conditions where both the elasticity and the fluidity have the proper relative magnitude to achieve successful annealing are low water (i.e. <8% weight) and temperatures of the order of 120–125°C. The annealing process is continuously monitored by an optical conoscopic technique to be discussed below.

Once the sample appears to be in the monodomain state, the temperature is gradually lowered to 80–90°C and the "removable spacers" (see Fig. 1) are removed from the clamped sample. The entire assembly is placed in water at approximately 80°C for a fixed length of time. The spacers are reinserted, the sides are clamped, effectively sealing the sample, the assembly is then removed from the water, and the entire assembly is allowed to equilibrate for 4–6 hr at approximately 80°C. Experimentally we find that this time is sufficient for the optical properties to become uniform over the dimension of the sample. The optical conoscopic monitoring technique is employed for this purpose. Experiments on these sealed samples are then carried out as described below. The precise water content of these samples is deter-

mined after all experiments are completed by a gravitometric method like that of Janiak, et al. (12).[1]

The assembly is taken down to room temperature, the clamps are removed, and the sample is opened. A small amount of lipid from the center of the sample is then scraped out and weighed. In view of the fact that the lipid can exchange water with the atmosphere, the weighing was repeated several times over a period of 30 s–10 min after the sample was opened. If the weight varied with time, the sample weight was taken to be that value obtained by extrapolation back to zero time. The dry weight was determined in a similar manner after the same lipid was maintained between 90 and 95°C under vacuum of less than 1 μm. The weight fraction of water in the original sample is determined from these two measurements. The largest uncertainty results from the fact that the amount of sample measured is small ($\lesssim$0.01 g). Consequently, our water concentrations are probably no better than $\pm\sim 1\%$ weight accurate.

Since the annealing procedure involved maintaining the sample at $\approx +125$°C for some hours, thin layer chromatography was done on lipid extracted from several samples after all measurements were completed. In all cases for which good macroscopic single domains were observed, the TLC results were identical to those obtained before the annealing procedure. Also, we will show below that for pure DPPC in excess of 20% water by weight, the temperature of the liquid-crystal-to-gel transition is identical to the published values for pure DPPC-water mixtures (9).

Fig. 2 indicates the submersion time necessary to achieve a particular weight fraction of water (measured at 25°C) in the aligned system. Three points should be noted. Firstly, that there appears to be some sort of barrier to adsorption of water at 20% weight. In view of the uncertainties in the dry weight determination, it was not possible to explore the steep part of the curve in detail. Secondly, the time necessary to obtain a specific weight fraction between 20% and 30% is very critical and it is not possible to use the time of immersion to control the water content in this region. Thirdly, there is some sort of saturation behavior at 30% water. Samples immersed for longer periods exhibited visible deterioration in alignment around the edges. This may result because during immersion the water content is larger at the sample edges than in the center.

DPPC + INORGANIC SALTS Both NaCl and $CaCl_2$ were incorporated in oriented lamellae starting from relatively anhydrous DPPC ($\leq\sim 1\%$ water) and a 0.1 M solution of either salt. Between 2 and 5% water was added to the pure lipid in the form of these salt solutions and alignment was achieved at approximately the same temperature as for the pure lipid. Extra water was added to aligned lecithin samples by immersing them in a 0.1 M solution of either NaCl or $CaCl_2$ at 80°C. The maximum water uptake with the NaCl solution before the sample alignment began to deteriorate around the edges corresponded to about 30% weight water to total sample weight. This is identical to the observation for pure water. The apparent barrier to water penetration shown to occur at 20% water in Fig. 2 is unchanged. In contrast, on swelling with 0.1 M $CaCl_2$ solution, the barrier appears at 10% water. The maximum uptake of $CaCl_2$ solution before alignment deterioration at the sample edges is only 25%, in contrast to 30% for pure water.

DPPC + CHOLESTEROL Cholesterol is incorporated into the samples by first dissolving both the DPPC and an appropriate amount of cholesterol in a small amount of benzene or other suitable solvent. This solution is quickly frozen and the solvent is pumped off. The dry mixture is allowed to thaw and then hydrated to approximately one water molecular per hydrophilic group. This could be achieved either by exposing the dry mixture to a N_2 atmosphere of controlled relative humidity or by physically adding the correct amount of water to the mixture. In the former case the length of time for exposure varies with the

[1] D. M. Small. Personal communication.

temperature, the actual relative humidity, and less well-defined variables such as the surface-to-volume ratio of the mixture. For high relative humidity and an exposure temperature of about 50°C, suitable hydration is typically achieved in less than 1 hr. If water is directly added to the sample, it is necessary to allow sufficient time for the sample to become homogeneous. However, this can occur in minutes at temperatures of the order of 50°–70°C. Oriented samples are prepared from these mixtures in the same way as for pure DPPC, except that the temperature at which annealing occurs depends on the mole fraction of cholesterol to lipid plus cholesterol. For fractions of 0.20, 0.33, and 0.50 these temperatures are ~70°C, ~50°C, and ~45°C. In each case higher temperatures obtain a mosaic-like texture that does not anneal away. Samples containing a cholesterol fraction of 0.50 and low water appeared to exhibit phase separation. Two types of regions with optical properties characteristic of 0.33 cholesterol fraction and a pure cholesterol fraction could be observed microscopically and studied by the conoscopic method. Water can be added to these samples by the submersion method below but near the respective alignment temperature. Maximum water uptake before alignment deterioration was observed to be 25% by weight water.

DPPC + ANTIBIOTICS OR CHLOROPHYLL *a* Anhydrous mixtures of DPPC and chlorophyll *a*, either of the carrier antibiotics, nonactin or valinomycin, or the pore-forming antibiotic, gramicidin A, were formed as described above for cholesterol. Antibiotic to DPPC mole fractions below 1% always obtained single-phase regions during the alignment and water swelling processes. At higher concentrations the different antibiotics exhibited more complicated phase separation effects that were not carefully studied. Samples hydrated to between 2 and 5% water exhibited partial alignment effects between 70 and 80°C. However, complete alignment resulting in optically clear samples were only obtained on heating to the annealing temperature of the pure DPPC (~125°C). The maximum hydration possible before the sample alignment started to deteriorate at the edges corresponds to water weight fractions of 24% of the total sample weight.

Samples containing less than 0.6 mol % of chlorophyll *a* to DPPC were aligned by annealing at ~125°C. These did not show any evidence of separation into regions of differing chlorophyll concentrations at any temperature between 120°C and room temperature for water concentration less than approximately 23% by weight. For greater water concentrations aggregates of chlorophyll-rich regions seemed to appear. Also, for chlorophyll concentrations above ~0.6 mol %, chlorophyll-rich regions could be observed optically under a variety of conditions. For samples as thick as 50 μm, chlorophyll in excess of approximately 1 mol % rendered the samples so opaque that conoscopic experiments could not be done.

Experimental Methods

CONOSCOPY In view of the fact that the lamellar lipid phases are optically anisotropic (i.e. indices of refraction for different polarizations are not identical), the conoscopic technique illustrated in Fig. 3 is an excellent way to monitor sample alignment. The microscope objective focuses a laser beam to a point somewhere near but not inside the sample. Neglecting diffraction effects, the optical ray connecting the point focus to any viewing point on the screen corresponds to one direction for light propagation through one point in the sample. In view of the sample birefringence, light polarized by the first polarizer (the x-axis is defined as perpendicular to the plane of polarization) will be depolarized for some propagation directions and not for some others. The result is that for a uniform medium with uniaxial direction along the z axis, the two crossed polarizers obtain complete extinction for light propagating in the two planes xz and yz and also for certain angles of propagation with respect to normal or z direction. The pattern of a dark cross and a concentric ring shown in Fig. 1*a* and *b* of Powers and Clark (8) corresponds to this case. If ϕ defines the angle between the propaga-

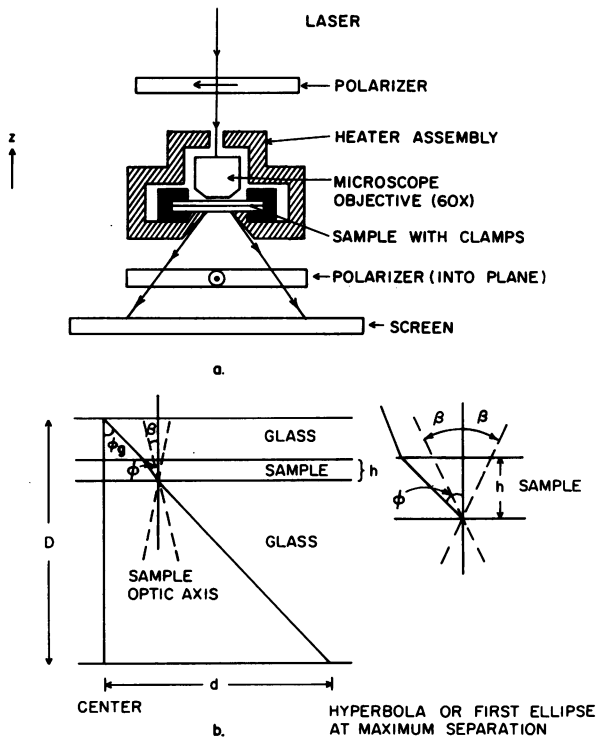

FIGURE 3 Schematic diagrams and experimental geometry of the conoscopic interference technique. Top, experimental apparatus; bottom, geometry for birefringence measurement.

tion directions responsible for the dark circle and the normal to the glass slide (see Fig. 3*b*), the index of refraction difference corrsponding to the uniaxial birefringence ($n_x = n_y \neq n_z$) is

$$n_z - n_x = \lambda n(n^2 - \sin^2\phi)^{1/2}(h\sin^2\phi)^{-1}, \quad (1)$$

where h is the smectic thickness; since $|n_z - n_x| \ll 1$, n is the average of the two. In actual practice the angle was not measured in air but inside a glass cone of index $n_g = 1.514$. In that case $\sin^2\phi$ is replaced by $n_g^2 \sin_g^2 \phi_g$ where ϕ_g is the angle between the propagation direction in the glass and the sample normal. The angles ϕ_g are smaller than for ϕ, allowing a smaller optical aperture. We neglect this point in the following.

If, however, the sample was not uniformly aligned, or if there were local defects, the polarization properties of the light passing through different parts of the sample would be distorted and the nonuniformity or defect would be visible. Note that since the dark cross can be made to rotate about its center by rotating both polarizer and analyzer together about the z-axis, the entire illuminated region of the sample can be scanned. In the case of an optically biaxial sample ($n_x \neq n_y \neq n_z$) and some orientation of the crossed polarizer-analyzer combination, the cross in the center opens up into two hyperbola-like figures and the circle becomes elliptical. This is shown in Fig. 1 of Powers and Clark (8). The angular distance of closest approach between the two hyperbola-like figures (2β) varies with the orientation of the crossed polarizer-analyzer around the uniaxial axis. When the separation is a

maximum, the index of refraction difference that distinguishes uniaxial from biaxiality is given by (13)

$$n_y - n_x \simeq |n_z - n_x| \beta^2, \tag{2}$$

where now

$$n_z - n_x \simeq \lambda n(n^2 - \sin^2 \phi_x)^{1/2}[h(\sin^2 \phi_x - n^2\beta^2)]^{-1}. \tag{3}$$

The angle ϕ_x is measured between the center of the pattern and the first dark ellipse in the same plane a β (Fig. 3*b*). Measurement of these conoscopic patterns is a relatively precise way to measure small index of refraction anisotropies. On the other hand, simple visible observation is sufficient to determine the homogeneity of the sample and to distinguish between uniaxial and biaxial phases. Both the annealing and subsequent cooling cycle are continuously monitored by this technique.

Conoscopic measurements of birefringence were always made as a function of decreasing temperature. Empirically it was found that aligned samples could be maintained aligned if the temperature was decreased, but on heating, alignment was often destroyed. Powers and Clark (8) explain this in terms of an "undulative instability" that follows from the fact that the lipid bilayer thickness generally decreases on raising the temperature. Although we found that if the temperature is raised very slowly (<0.01°C/min) it is often possible to maintain sample alignment, it was not practical to measure the birefringence as a function of increasing temperature.

RESULTS

DPPC

Conoscopic results for the monohydrate of DPPC were contained in Fig. 2 of Powers and Clark (8). Similar results for other water concentrations are described in Fig. 4.

The monohydrate sample discussed by Powers and Clark (8) was monitored as it cooled from the annealing temperature ~125°C. Although no biaxiality was observed at high temperatures, the uniaxial birefringence increased steadily with decreasing temperature. At approximately 80°C there was a sudden increase in the amount of scattered light and an accompanying decrease in the light transmitted by the sample. This was interpreted as the onset of a two-phase region, in which sample inhomogeneity gives rise to a turbidity that scatters light. Similarly, Fig. 4*a* shows a weaker but nevertheless measurable temperature dependence for $n_z - n_x$ in the uniaxial region of the phase diagram for the 13% sample. The onset of scattering, as indicated by the arrow at ~62°C, marks the high-temperature end of a two-phase region for this water concentration. Similar data (not shown) for a 16% water sample indicates that a two-phase region starts at ~54°C for this water concentration. On further lowering of temperature of the 13% sample, turbidity abruptly decreases and an optical biaxiality becomes measurable at 52°C. For the monohydrate, Powers and Clark observed this at 72°C. For a 16% water sample the turbidity decreases abruptly at 48°C, but no biaxiality could be detected.

Fig. 4*b* shows the birefringence data for a 19% water sample. Note the two sets of data above 54°C. These indicate that the sample was macroscopically inhomoge-

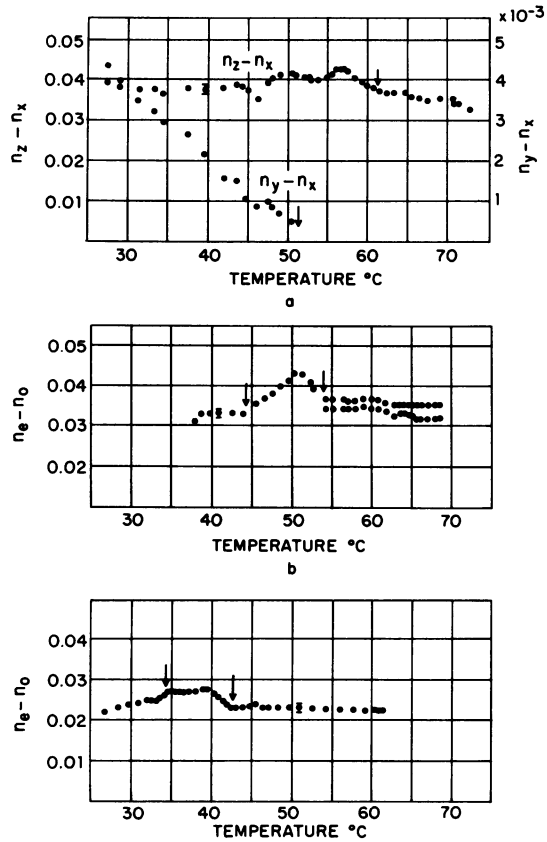

FIGURE 4 Birefringence data as a function of temperature for oriented samples of DPPC containing water. Arrows indicate changes in transmitted intensity. *a*, 13% weight water; *b*, 19% weight water; *c*, 21% weight water.

neous. A sharp line dividing the sample into two regions was observed and one set of data corresponds to one side of the line and the other to the opposite side. Gary-Bobo et al. (14–17) observed a transition at approximately 20% water in egg yolk phosphatidylcholine that they ascribed to a change from a bent to an extended conformation of the polar group. Since both conformations would appear uniaxial by the conoscopic technique, the only optical property that might distinguish them would be a sudden change in the magnitude of the birefringence. Our observation would be consistent with the coexistence of two different uniaxial phases at slightly different water concentration. The magnitude of the uniaxial birefringence shown in Fig. 4*c* for 21% water is almost identical to the high temperature value for one of the two sets of data in 4*b*. There is a change in transmitted intensity indicated in 4*b* at ~54°C that we interpret as the top of a two-phase region. A second change in transmitted intensity at ~44°C is not interpreted. Biaxiality could not be observed in this sample at low temperatures.

The data in Fig. 4*c* for a sample containing 21% water is independent of temperature until 41.8°C, where there is a sudden change in sample turbidity. This coincides with the well-known gel transition temperature. Between 40°C and 34.5°C, the sample remains slightly turbid, but below 33°C, it becomes clear and optically uniaxial. Results for samples containing 30% water (not shown) were similar to those described for 21%.

DPPC + Ionic Salts

Conoscopic measurements of the birefringence of DPPC containing ≈2% of a 0.1 M NaCl solution were contained in Powers and Clark, (8) Fig. 3. The principal difference between the DPPC plus NaCl and DPPC plus pure water was a decrease in the magnitude of the biaxial birefringence. Fig. 5*a* for 20% weight of 0.1 M NaCl is only slightly different from the result shown in Fig. 4*c* for 21% water. Although the result shown in 5*b* for 10% weight solution of 0.1 M $NaCl_2$ solution is also similar to these two, it is strikingly different from the result in Fig. 4*a* for 13% water.

DPPC + Cholesterol or Antibiotics

Birefringence measurements were done on samples of ~33 mol % cholesterol to lipid for water concentrations of ~5%, ~13%, and ~25% weight water to total sample weight. Data for the ~5% water sample are shown in Fig. 6*a*. The appearance of turbidity, indicated by the arrow, at approximately 78°C is near to the temperature at which the gel transition was observed in pure DPPC monohydrate (8). For lower temperatures, however, the cholesterol sample did not exhibit any measurable biaxiality. Samples of 13% and 25% water do not show any evidence for the gel transition, but for the 25% water sample, some turbidity at ~28°C suggests a phase transition of some kind. Aside from this, the birefringences for these two water concentrations are almost completely independent of the temperature. The birefringence on the other hand is nearly two times larger than for the pure DPPC at comparable water concentrations and temperature.

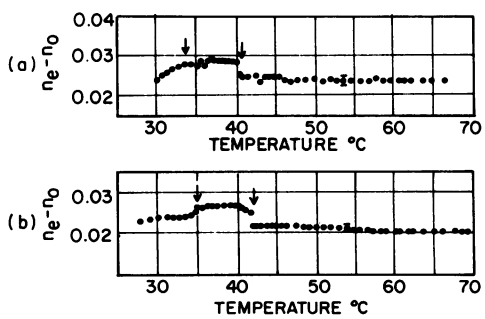

FIGURE 5 Birefringence as a function of temperature for DPPC samples containing fixed amounts of a salt-water solution. *a*, 0.1 M Nacl, 20% weight of solution to total sample; *b*, 0.1 M $CaCl_2$, 10% weight solution to total sample.

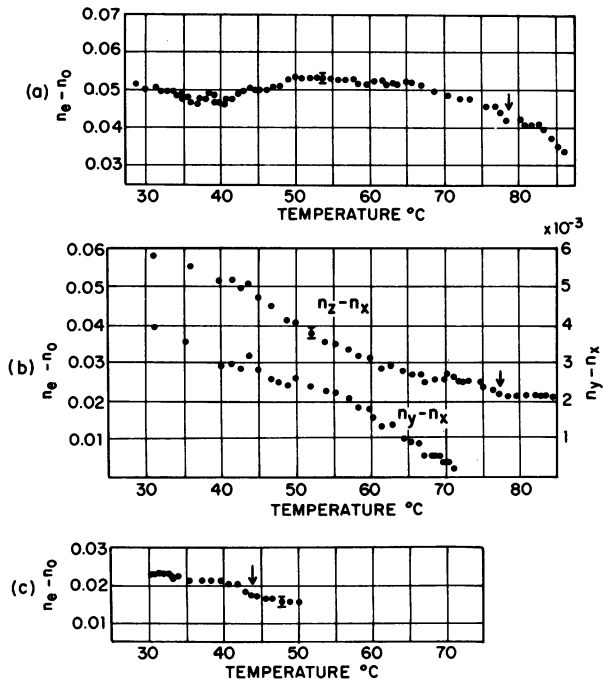

FIGURE 6 Birefringence as a function of temperature for *a*, a mixture of 33% mole fraction cholesterol in DPPC, i.e. one molecule of cholesterol to two lipid molecules, with less than 5% weight water to total sample; *b*, a mixture containing 1 mol % of gramicidin-A to DPPC at less than 5% water; and *c*, at 24% water.

Birefringence data for the monohydrate with gramicidin A are shown in Fig. 6*b*. It exhibits a biaxial birefringence below ~77°C, similar to that of the pure DPPC. The uniaxial anisotropy, however, is a strongly increasing function of decreasing temperature for the gramicidin A sample, while for the pure lipid it was essentially constant between 80°C and room temperature. For higher water concentration the data are shown in Fig. 6*c*. The turbidity that indicated the onset of the gel transition occurs at ~43.5°C, while for the pure lecithin it was at ~41.8°C. There is no evidence for a lower temperature phase transition (near 33°C) that exists in pure DPPC at high water concentrations. A similar observation was made by Chapman et al. (18).

Current versus applied voltage measurements were done by one of us (L.P.) in collaboration with P. Mueller on a DPPC sample containing 0.33 fraction cholesterol, ~1 mol % gramicidin A, and ~6% weight 0.1 N NaCl solution at 25°C. The results were identical to what is predicted by assuming that the stacked multilamella is electrically equivalent to $\sim 10^5$ individual gramicidin A-containing bilayers in series. The electrical properties of the individual bilayers are known from previous measurements (19).

Birefringence measurements on the samples containing either nonactin or valinomycin do not exhibit any biaxial behavior at low water. The uniaxial birefringence is

slightly smaller than for pure lecithin, and while the gel transition is detectable, the change in the birefringence at the gel transition is smaller for the samples containing these antibiotics than for the pure samples. Results for high water are not significantly different from those for gramicidin A in DPPC, and the barrier to water penetration that occurred at ~20% weight for pure DPPC occurs at ~15% weight when any of these antibiotics are incorporated.

DPPC + Chlorophyll a

Although the uniaxial birefringence at both high and low water concentrations of DPPC containing less than 0.6 mol % chlorophyll *a* was identical to that of pure DPPC, there was no evidence for the biaxial birefringence observed in the pure sample at low water and temperature. Absorption spectra of aligned samples containing less than 0.6 mol % chlorophyll *a* exhibited the expected spectral features (20–22).

Detailed study of the polarization properties of the chlorophyll *a* absorption band at ~670 nm were carried out as by Hoff (23). For incident light of arbitrary polarization $\hat{e}$, the quantum mechanical probability for a transition from some state α to β is most generally expressible in terms of a second rank tensor $P_{ij}(\alpha \rightarrow \beta)$; the transition probability, or finally the absorption cross-section is given by

$$\sum_{i,j} (\hat{e})_i P_{i,j}(\alpha \rightarrow \beta)(\hat{e})_j.$$

Only for very special cases, when the absorption process is completely polarized, $P_{i,j}$ has a specially simple form and the transition probability can be written $|(\mathbf{M} \cdot e)|^2$ where $\mathbf{M}$ is a vector defined by the molecular wave functions. For empirical purposes it is important to recognize that this form is a special case and not generally applicable. In the present case we assume the 670–680 nm band of chlorophyll *a* has this simple form and that $\mathbf{M}$ has some fixed relation to the lamellar structure, as shown in Fig. 7. Incident light polarized along $\hat{e}$ will then have an absorption probability proportional to $A \equiv |\mathbf{M} \cdot \hat{e}|^2$ where $|\mathbf{M} \cdot \hat{e}| \sim \sin\alpha \cos\phi \cos\theta \cos\psi + \cos\alpha \sin\phi \cos\psi + \cos\phi \sin\theta \sin\psi$. Note that, in addition to the assumption mentioned above, if we assume the molecule itself is not rotating and that all molecules in the sample have the same orientation, the observable absorbance will also be proportional to A. If the molecule were rotating or if there were some distribution of orientations, we would require the average of A over the appropriate angular distributions. Measurement at $\alpha = 90°$ or $\mathbf{M} \sim \cos\phi\cos(\theta - \psi)$ is most easy. To confirm our assumptions on the form of A, we measure the total absorbance A and plot $\sqrt{A/\cos\psi}$ versus $\tan\psi$. The above form should yield a straight line of the form $\cos\theta + \sin\theta\tan\psi$. Fig. 8*a* shows the result of this plot for two separate monohydrate samples. Since the angle θ, and thus the value of $\cos\theta$ and $\sin\theta$ are arbitrary, being fixed by the arbitrary choice of the origin for ψ, it is purely fortuitous that two samples give $\theta = 53°$ and 68°, respectively. Nevertheless this curve proves that for the monohydrate there is long-range order in the angular orientation of the chlorophyll absorption dipole. Fixing $\psi = 0$ and plotting $\sqrt{\mathrm{M}/\cos\alpha}$ versus $\tan\alpha$, we obtain the straight line in Fig. 8*b*. The

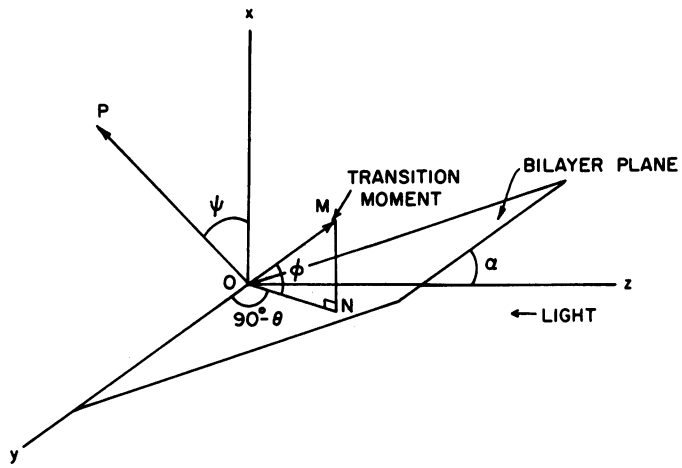

FIGURE 7 Geometry of sample and transition moment for polarized absorption spectroscopy. Light propagation is along the z-axis. Rotation of the bilayer plane about the y-axis is specified by α; θ is in the bilayer plane; **ON** is the projection of **OM** on the bilayer plane, and the optical polarization is specified by a unit vector **P** making an angle ψ with the x-axis in the x-y plane.

ratio of the intercept on the vertical axis to the slope is $\tan\phi/\cos\theta$ and from the two samples we can obtain values of $\phi = 23° \pm 2°$ and $25° \pm 2°$. Although this angle is considerably smaller than values quoted for other systems, it is not surprising since at low water content the hydrophilic part of the chlorophyll group is squeezed into a relatively small (~6–10 Å) water bilayer.

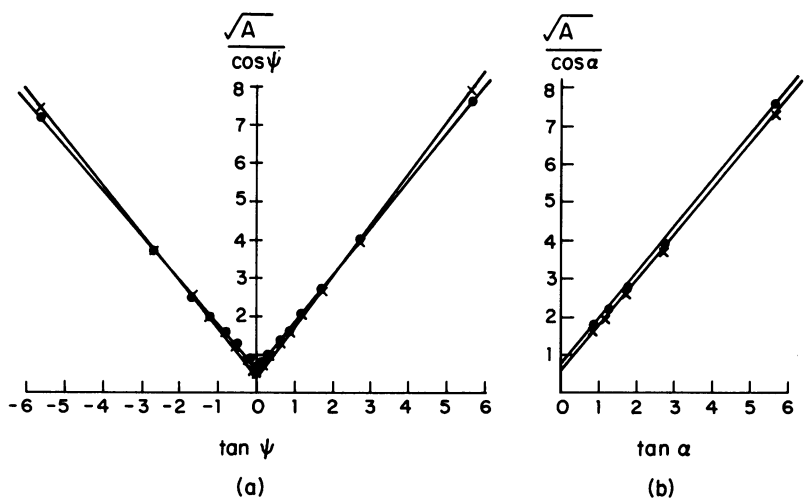

FIGURE 8 Polarized absorption data for two oriented DPPC monohydrate samples containing ~0.6 mol % chlorophyll *a*. See Fig. 7. *a*, ψ is the angle the polarization of the incident light makes with the x-axis in the x-y plane of Fig. 3 when $\alpha = 90°$; *b*, α is the angle the incident light makes with the bilayer plane when $\psi = 0°$.

For higher water content there was no detectable dichroism, indicating the absence of macroscopic order in the dipole orientation within the bilayer plane. There was also no detectable variation in absorbance with $\psi = 0$ for different α. This could be interpreted as either $\phi \simeq 35.2°$ (22) or ϕ is randomly distributed over a wide range of angles.

DISCUSSION

The strongest evidence in support of our contention that we have been able to add variable amounts of water to the aligned DPPC samples described by Powers and Clark (8) is the phase diagram that can be deduced from the optical conoscopic studies. This is shown in Fig. 9. Below approximately 20% water, for higher temperatures, the samples were all uniaxial and optically clear. On cooling, they became turbid at a temperature that varied with water concentration and that we interpret as the high-temperature boundary of a two-phase region. On further cooling the monohydrate and the 13% sample became clear and biaxial. The upper edge of this two-phase region can be followed to higher water content and above 20% water it agrees with the known gel transition temperature. On the other hand, between 16% water and 21% water the optical data cannot be interpreted below the gel transition. This region may be a two-phase region, or alternatively we may have failed to obtain

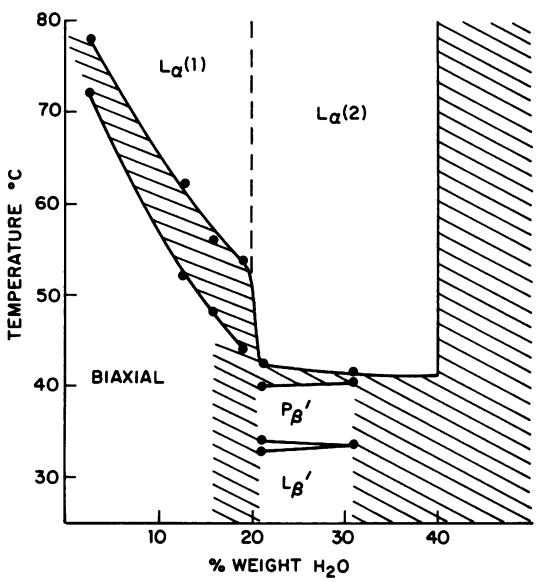

FIGURE 9 Optically deduced phase diagram for oriented mixtures of DPPC and water, as obtained from birefringence studies. The points show where sudden changes in the optical properties were observed. The slash marks indicate where the samples do not appear homogeneous. These are probably two phase regions. The boundary at 40% water was not established in our measurements (9).

macroscopic alignment. In excess of 20% water, phase transitions are indicated just where they have been measured to be by other techniques (12).[1]

This phase diagram is similar to the one first proposed by Chapman et al. (9) The two-phase region between the biaxial phase and $L\alpha(1)$ is qualitatively similar to that reported by Gottleib and Eanes (24), although there is some quantitative differences between their temperatures and ours. Janiak et al. (12)[1] have also obtained a phase diagram for DPPC that is essentially the same as shown here except for the higher water content region, and we have used their X-ray data to label the phases marked $P_{\beta'}$ and $L_{\beta'}$. The difference between $L\alpha(1)$ and $L\alpha(2)$ is not yet clear. Gary-Bobo et al. (14–17) did suggest such a phase boundary in egg yolk phosphatidylcholine, and our birefringence data (Fig. 4*b*), as well as the data on the rate of water uptake (Fig. 2), are consistent with some effect at 20% water, although it is not necessarily a phase transition. The principal new feature, not previously reported, is a macroscopic biaxial ordering for low water, below the gel. Nuclear magnetic resonance (25) on aligned samples support this and the suggestion made by Powers and Clark (8) that it was principally due to the polar group orientation. Other supporting evidence is that samples containing gramicidin A, which resides primarily in the hydrocarbon region of the bilayer, exhibit a biaxial birefringence similar to that of pure DPPC but a uniaxial birefringence that is altered. On the other hand, the phytol chain of chlorophyll *a* does not disrupt the organization of the hydrocarbon chains of lipids nearly as much as gramicidin A, but the chlorophyll samples do not exhibit a biaxial phase and their uniaxial birefringence is similar to the pure DPPC.

DPPC samples swelled with 0.1 M% NaCl yield results almost identical to those of DPPC swelled with pure water. One might note that that Gottleib and Eanes (26) studied the binding of various cations to the synthetic lecithin 1-octadec-9-enyl-2-hexadecyl-glycerophosphocholine and concluded that Na^+ does not bind to this material at high water. One might presume from the present measurements that the same is true for DPPC. On the other hand, the slight reduction in the gel transition at high water and the reduction in the biaxiality at low water supports the conclusion that Na^+ has been incorporated. The significant changes in the rate of water uptake, the birefringence of 10% water for samples swelled with $CaCl_2$, and raising of the temperatures at which the gel and $P_{\beta'}$ to $L_{\beta'}$ transitions occur supports the conclusion that Ca^{++} has been incorporated and suggests some binding.

Evidence that cholesterol has been incorporated is obtained from the several differences between the birefringence results for pure DPPC and that mixed with cholesterol. Especially significant here is the absence of measurable biaxiality at low water and a factor of two increase in the uniaxial birefringence at high water in comparison with pure DPPC at the same water concentration. Since the increase of uniaxial birefringence in pure DPPC accompanies those water or temperature changes that tend to decrease the area per molecule, it is reasonable to suppose that the 33 mol % cholesterol samples may have smaller area per molecule than pure DPPC. This would be consistent with a close-packed structure like that proposed by Rothman and Engleman (27).

The differences in the birefringence of the antibiotic containing DPPC and the pure DPPC samples demonstrated that the antibiotics were incorporated. Similarly, the experiments described above support the conclusion that chlorophyll *a* has been incorporated into aligned DPPC. For low water we also demonstrate a specific ordering of the chlorophyll *a* relative to the lipid bilayer. Our data for higher water content are consistent with Hoff's (23), although we claim that orientation of the chlorophyll *a* was not demonstrated. We also optically observed that chlorophyll *a* exhibited phase separation phenomena at concentrations in excess of ~0.6 mol %. This is consistent with the results of Lee (28).

In summary, we have demonstrated that monodomain multilamella DPPC samples as thick as 500 μm can be obtained at varying water concentrations and with various other membrane-associated molecules incorporated. We hope these samples may facilitate a variety of physical studies on lipid systems not otherwise possible with thinner samples.

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