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ELECTRON DIFFRACTION STUDY OF HYDRATED PHOSPHOLIPID SINGLE BILAYERS

EFFECTS OF TEMPERATURE, HYDRATION AND SURFACE PRESSURE OF THE "PRECURSOR" MONOLAYER

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SUMMARY

The molecular packing and phase transition of hydrated dipalmitoylphosphatidylcholine single bilayers are studied by electron diffraction, using an electron microscope equipped with a hydration stage. The phase transition and area per molecule are measured as functions of temperature, hydration and the surface pressure of the monolayer from which the bilayer is formed. The transition temperature of a bilayer agrees with calorimetric measurements on bulk lipid/water mixtures. The molecular packing of a bilayer corresponds to that of the precursor monolayer at a surface pressure of 47 dyne/cm.

INTRODUCTION

Because of its relevance to the biological membranes, the structure and the physical properties of phospholipid bilayers have been subjected to extensive study. The molecular packing of bulk lecithin/water mixtures as a function of both temperature and hydration has been studied by X-ray diffraction [1, 2]. X-ray diffraction studies of the phospholipid multilayers, either of the Langmuir-Blodgett type or of the concentric vesicle form, have also been reported [3, 4]. Using fully hydrated phospholipids, the phase transition between the ordered two-dimensional solid state and the less ordered liquid crystalline state was detected by various methods, including X-ray diffraction [1, 2], differential scanning calorimetry [5], magnetic resonances [6, 7] and dilatometry [8]. All of these measurements were made on bulk samples, and consequently the properties of a small area in a single bilayer are represented only as a gross average. Since electrons scatter strongly from thin layers, electron diffraction

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provides a direct method to determine the structure on a microscopic scale. With the development of hydration stages [9], the physical states of the specimen can be monitored with respect to hydration and temperature. In this paper, we present our electron diffraction results on the structure of dipalmitoylphosphatidylcholine monolayers and unsupported bilayers, both made by the Langmuir-Blodgett technique on electron microscope grids. The data are presented as a function of temperature, hydration and surface pressure of the "precursor" monolayer. In order to compare the structure of a bilayer and of a monolayer, we have extended the published data [10] on the pressure-area properties of surface monolayers at different temperatures.

MATERIALS AND METHODS

Chemicals

L-α-1,2-Dipalmitoylphosphatidylcholine was synthesized by the method of Robles and Van den Berg [11] as described in detail earlier [12]. Purity was confirmed with silica gel H thin-layer chromatography developed with a chloroform/methanol/7 M ammonia solvent (230/90/15; v/v/v). Dipalmitoylphosphatidylcholine was stored in chloroform in sealed ampoules under nitrogen at −50 °C, at a concentration of approximately 10 μmol/ml. Just prior to the experiments, the content of an ampoule was transferred to a Teflon-sealed screw-capped culture tube and diluted to approximately 1 μmol/ml. Phospholipid concentration was assayed by measuring inorganic phosphorous by a modification of the Fiske and SubbaRow method [13].

The buffered subphase used in the Langmuir trough was 10 mM NaCl (Baker), 0.1 mM EDTA (Mann), 2 mM histidine (Sigma), 2 mM TES (*N*-tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid) (Sigma), pH 7.4. Certain electron diffraction specimens were made with a subphase of 50 μM $CaCl_2$ (Fisher) and 2 mM Tris-(hydroxymethyl)-aminoethane (Fisher) pH 6.8. However, the different subphases had no effect on the diffraction patterns of dipalmitoylphosphatidylcholine.

Force area measurements

Force-area data for dipalmitoylphosphatidylcholine were measured with an automated Teflon Langmuir trough (Surface area 225 cm^2 (7.5×30 cm); 400 ml total capacity, mounted on a 1/2 inch stainless steel base plate). Dipalmitoylphosphatidylcholine (1 μmol/ml) in chloroform/methanol 9/1 (v/v) was applied to the surface with a precalibrated 20 ml Lang-Levy micropipet and the film was compressed at a rate of 5 cm^2/min. Surface tension (measured with a Cahn RG electrobalance) and surface area were both recorded on a Watanabe Multicorder. The area of the film was defined by the position of a Teflon barrier, whose motion was controlled by a servo mechanism coupled to the output of the electrobalance. After carefully lapping the trough and barrier contact surfaces with 40 micron grade aluminum oxide lapping film (The 3M Co.), high surface pressure data were obtainable with a slightly concave meniscus. Small variations in the force vs area curves were observed with respect to the concave-convex nature of the meniscus. At surface pressures in excess of 45–50 dyne/cm, the film ballooned out over the edge of the trough even when the meniscus was approximately flat. This phenomenon was also observed with a slightly concave meniscus at pressures greater than 60 dyne/cm. It was not until the meniscus was

concaved below the trough by at least 1–2 mm that pressures greater than 70 dyne/cm could be obtained.

Constant temperatures were controlled by running the experiments in a water-jacketed Lucite box and by running water through a glass coil in the Teflon trough both regulated by a Forma-Temp Jr. (model 2095) circulation bath. The subphase was mixed using a small Teflon bar magnet. Compression of the monolayer films was started 5–10 min after the addition of dipalmitoylphosphatidylcholine to the clean surface and the lift-off (see Results section) occurred approximately 10 min after the compression onset, thus giving ample time for the solvent to evaporate form the air-water interface (15–20 min).

Formation of bilayers

Various methods were used to form lipid films on electron microscope specimen grids. These methods have been described elsewhere [14]. Here, we consider only the methods of interest to this work.

An unsupported bilayer is one which forms over empty grid squares when the grids are passed through the phospholipid monolayer on the water-air interface of the Langmuir trough. The lipid chains of the surface monolayer were presumably "zipped up" to form a bilayer on the grid squares during the passage. A monolayer "deposit" is one which deposits on the surface of the Formvar coating of the grid by dipping the coated grids in the same manner described above. The Formvar coating is "hydrophobic" unless it is cleaned by glow discharge. Surface film spread on the water-air interface of the Langmuir trough is referred as to a precursor monolayer.

All the lipid films were made at 10 °C under saturated water vapor. The grids carrying the wet films were immediately transferred into a humidity box to be mounted into the specimen holder, which was then inserted into the hydration stage of the electron microscope.

Electron diffraction

The hydration stage made for a Siemens Elmiskop IA has been described elsewhere [15]. The temperature of the stage and its internal water reservoir could be controlled via a thermoelectric module. Thus, the specimen was under the corresponding saturated water vapor pressure at all temperatures. In controlled hydration experiments, an external water reservoir was used and its temperature adjusted to control the percentage hydration of the specimen.

The radiation damage to the specimen was minimized by lowering the beam current by using a small (10 μm) condensing aperture, and by recording the diffraction pattern with a sensitive X-ray film [16]. Unexposed, virgin areas of the specimen were used whenever patterns were recorded. All patterns were recorded at 100 kV accelerating voltage. The selective diffraction area was about $80/\mu m^2$ as limited by the small cross-sectional area of the beam.

RESULTS

1. *Force vs area temperature characteristics of surface monolayers*

The force vs area (π vs A) isotherms of dipalmitoylphosphatidylcholine monolayers are plotted in Fig. 1. The area per molecule is calculated from the amount

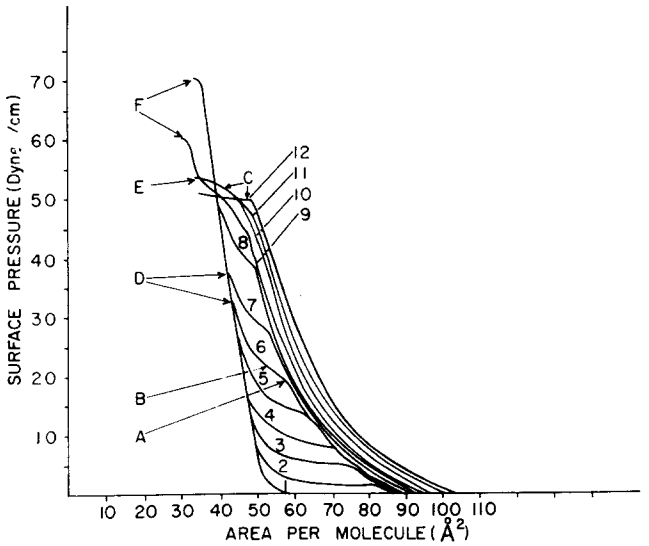

Fig. 1. Dipalmitoylphosphatidylcholine force vs area isotherms. Isotherm temperatures are numbered with increasing temperature: 1, 9.0 °C; 2, 15.5 °C; 3, 19.0 °C; 4, 22.8 °C; 5, 25.5 °C; 6, 29.2 °C; 7, 33.8 °C; 8, 37.2 °C; 9, 41.1 °C; 10, 41.7 °C; 11, 42.8 °C; 12, 44.0 °C. The letters indicate: (A) the boundary between the intermediate phase transition region and the fluid state, (B) the inflection point or region of maximum compressibility, (C) the liquid collapse pressure, (D) the boundary between the intermediate phase transition region and the solid state, (E) the point of resolidification after the liquid collapse and (F) the solid collapse region.

of phosphate added to the surface. The point at which the isotherm starts to show a non-zero pressure is termed "lift-off". From this point on, the monolayer is seen to behave as either a solid ($T < 12.8$ °C) or as a liquid ($T > 12.8$ °C). In the liquid state, a kink (Point A in Fig. 1) can be seen in the (π vs A) isotherms at temperatures below 41.8 °C. This point marks the beginning of an intermediate or phase transition region which terminates as the curve superimposes with the completely condensed or solid isotherm (Points D in Fig. 1). In this region, the inflection point (Point of maximum compressibility, B, in Fig. 1) can be observed up to a temperature just below 41.8 °C. At temperatures below 37.5 °C, solid monolayers collapse at pressures in the region of 70 dyne/cm (Point F in Fig. 1). At temperatures 42.0 °C, the monolayers do not solidify, and they collapse in the region of 50 dyne/cm (Point C in Fig. 1). At temperatures of 38.5 °C to 41.8 °C, with a barrier compression rate of 5 cm^2/min, it appears that both the liquid collapse and the solid collapse can be seen (Point E in Fig. 1).

To draw an analogy to a three-dimensional system, we may take the critical temperature of the monolayer as that temperature where the transition region of the pressure area isotherm no longer has a zero slope. Because dipalmitoylphosphatidylcholine monolayers do not have a zero slope, Phillips and Chapman [10] defined the critical temperature in monolayers (for dipalmitoylphosphatidylcholine particularly) as "that temperature above which π vs A curves no longer show an inflection". Using a surface trough in which the temperature could not be raised above 35 °C, Phillips and Chapman suggested (by extrapolation) that the critical temperature for dipalmi-

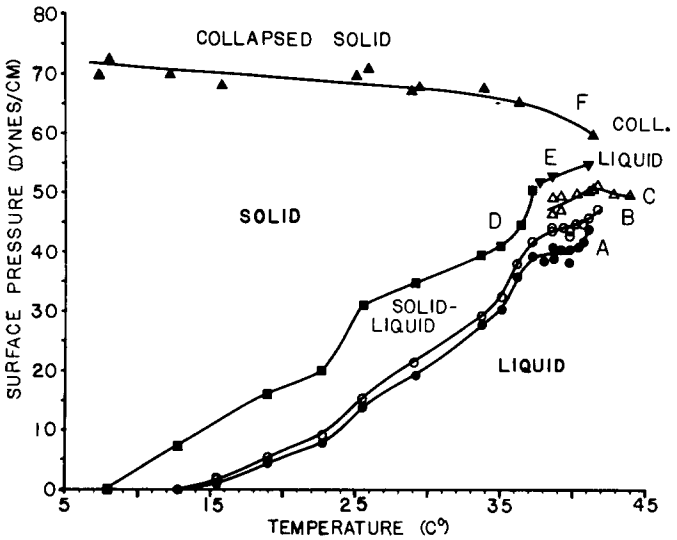

Fig. 2. Dipalmitoylphosphatidylcholine pressure vs temperature phase diagram. Points on curves A B, C, D, E, and F correspond to isotherm points represented by a corresponding letter in Fig. 1.

toylphosphatidylcholine is close to 41 °C and pointed out that this is analogous to the transition temperature T_c for dipalmitoylphosphatidylcholine bilayers. We have extended the π vs A data beyond 41 °C and up to the region of 70 dyne/cm. Our data show that although the solid-liquid transition region is no longer distinguishable at temperatures above 39 °C, the inflection point can still be followed up to 41.8 °C, which would be the critical temperature according to Phillips and Chapman's definition.

Compared to a three-dimensional system, the two-dimensional system has an additional phase, the collapsed phase. A surface pressure vs temperature phase diagram for a dipalmitoylphosphatidylcholine monolayer was constructed from force vs area data (Fig. 2). Curves A, B, C, D, E, and F correspond to isotherm points represented by a corresponding letter in Fig. 1.

2. *Effect of monolayer surface pressure on the resultant bilayer*

Bilayers and deposits (monolayers on Formvar) were made from monolayers at surface pressures from 10 to 70 dyne/cm. Below the transition temperature T_c of the bilayer, the diffraction patterns of these films are very similar. A typical diffraction pattern showing a hexagonal arrangement of arcs is shown in Fig. 3. The structure analysis of this pattern will be reported elsewhere [14]. The area per molecule of a two-dimensional hexagonally packed lattice is calculated in the following manner. The interchain distance is equal to $(2/\sqrt{3})d_{100}$ where d_{100} is the (100) spacing. Assuming that the hydrocarbon chains are normal to the bilayer surface (Hui, S. W., in preparation), the area per phospholipid molecule on a bilayer surface is then $(4/\sqrt{3})$ d_{100}^2. Fig. 4 shows the area per molecule at 25 °C as a function of the surface pressure of the precursor surface monolayer. In comparison to the π vs A curve of the precursor monolayer, the area per molecule of the resultant bilayers and deposits are less depen-

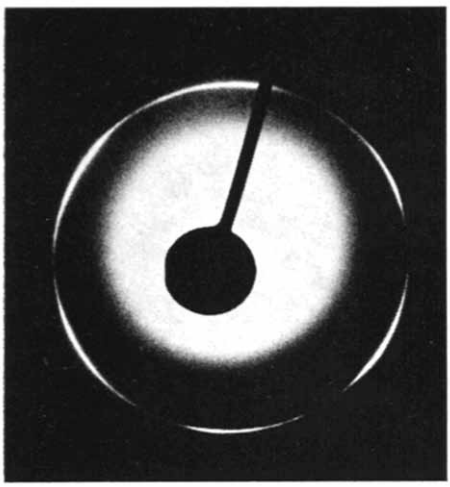

Fig. 3. An electron diffraction pattern of a wet, unsupported dipalmitoylphosphatidylcholine bilayer. The (100) spacing is 4.25 Å at 30 °C. (110) and (200) reflections can be seen from the original film.

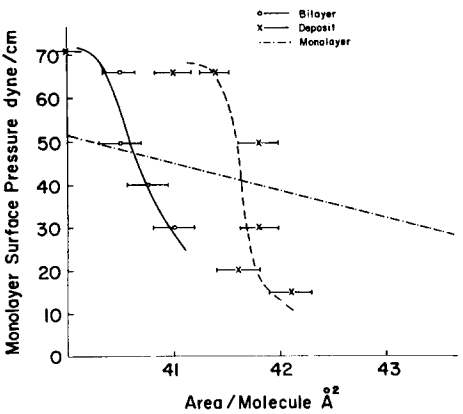

Fig. 4. The area per molecule of dipalmitoylphosphatidylcholine bilayers and deposits at 25 °C as functions of the surface pressure of the precursor monolayer. A portion of the force area isotherm of the precursor monolayer at 25 °C is also plotted in for comparison.

dent on π. The area per molecule of a deposit is invariably larger than that of a bilayer. Since in most cases the packing of the precursor monolayer is looser than that of the resultant films, it seems that the Formvar substrate tends to impede the repacking of films after they are formed.

At pressures less than 30 dyne/cm, it is very difficult to form unsupported bilayers. However, deposits can be picked up from a monolayer at a surface pressure as low as 10 dyne/cm. It should be noted that at the pick-up temperature of 10 °C the precursor monolayer is in a condensed state at all pressure ranges above 3 dyne/cm. Attempts to form bilayers and deposits from monolayers in the liquid state have not been successful. The pattern of a film picked up from a collapsed surface layer is usu-

ally stronger but more diffused, and the 6-fold symmetry degenerates into 2-fold. These features suggest that the collapsed film consists of several overlaying layers (Hui, S. W., in preparation).

3. *Effect of temperature on bilayers*

The spacings of the bilayer expand slightly with temperature until the sharp patterns disappear and a diffuse ring of a spacing approximately 4.4 Å is obtained (Fig. 5). This indicates that the structure of the bilayer has passed through a phase transition from a hexagonal packing to a liquid-crystalline structure. For all fully hydrated bilayers this phase transition takes place abruptly at 41.5 °C. The spacing as

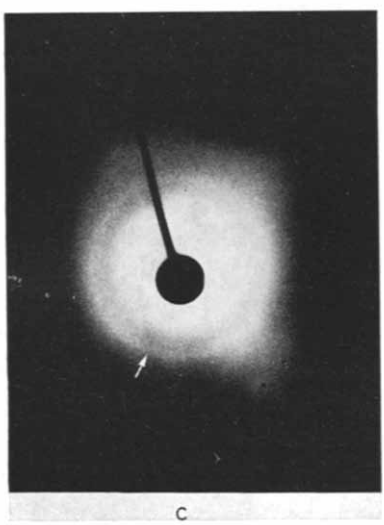

Fig. 5. An electron diffraction pattern of a wet, unsupported dipalmitoylphosphatidylcholine bilayer at 44 °C showing a diffuse ring (arrowed) at a spacing of 4.4 Å.

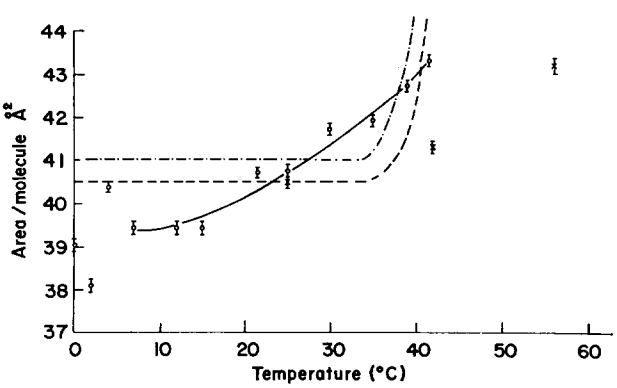

Fig. 6. The thermal expansion of dipalmitoylphosphatidylcholine bilayers at wet (○) and dry (×) states. The isopiezic curves of the precursor monolayer at 45 (- · · · -) & 47.5 (- - -) dyne/cm are also drawn in for comparison.

a function of temperature below the T_c is plotted in Fig. 6. For dried bilayers, the phase transition is still not seen at a temperature as high as 62 °C. This result corresponds with the phase diagram obtained by X-ray diffraction of a mixed lipid/water system [1] showing a similarity between the bulk system and a single bilayer. The T_c value for the fully hydrated bilayer specimen agrees with that measured by differential scanning calorimetry [5], spin label EPR [7] and dilatometry experiments [8] on multilayer systems.

Phase transition of dipalmitoylphosphatidylcholine monolayers deposited on Formvar may occur at a lower temperature depending on the pressure of the precursor monolayer. A diffuse diffraction ring similar to that in Fig. 5 was observed from a deposit picked up from a precursor monolayer at 10 dyne/cm and viewed at 24 °C. However, deposits picked up from a precursor monolayer at 15 dyne/cm showed sharp diffraction patterns at 24 °C. Apparently, the deposit retains certain properties of its precursor surface monolayer.

DISCUSSION

With the electron diffraction techniques, we were able to obtain microscopic structural information from a single bilayer or monolayer deposit. We are now in the position to compare the structural properties of bilayers to those of the precursor surface monolayers and of multilayers. The physical parameters we consider here are temperature, hydration, the surface pressure of the precursor monolayer and the molecular packing conditions. We shall first discuss the error in the measurement of area per molecule, then we shall compare the measurements of other parameters for monolayers, bilayers and multilayers.

In comparing our force vs area curves of the monolayer to those in reference 10, we found discrepancies in both the lift-off (90 $Å^2$/molecule at 26 °C, as compared to 105 $Å^2$/molecule in reference 10), and in the "limiting area" at 50 dyne/cm (40 $Å^2$/molecule as compared to the 44 $Å^2$/molecule). Some of the discrepancies found in the literature with regard to lecithin force vs area curves have been attributed to the hygroscopic nature of lecithin in the liquid-crystalline phase used for weight measurement [10]. This particular argument does not apply to our force-area data, since the lecithin concentration was determined by phosphate analysis. One major possible cause for error in our force vs area curves which would decrease the area per molecule would be the increase in working trough area due to a slight concavity of the meniscus necessary for high pressure force area curves. However, the meniscus error has been estimated to be only 0.70–1.2 % of the area. The force vs area curves did not vary more than ± 1 $Å^2$/molecule.

The area per molecule (A) for bilayers is calculated assuming that all the chains are perpendicular to the bilayer surface. For multilayers, this structure is said to be true only for dried specimens [1, 2, 3]. The calculation of the surface area per molecule in a wet multilayer structure was based on the layer thickness measurement and the specific gravity of the lipid, and a tilt angle ranging from 28° to 32° [1, 2] between the chains and the normal to the layer surface was proposed. However, from a symmetry argument, we have shown (Hui, S. W., in preparation) that this angle is zero for a single hydrated bilayer. Thus, the calculation of A in the previous section needs no normalization.

The spacings of bilayers and to a lesser extent of deposits are insensitive to the pressure of the precursor monolayer. It is possible that, as soon as it is freed from the constraint in the trough, the molecules in a bilayer tend to reach their lowest energy configuration within the new boundary conditions. For a deposit, on the other hand, the van der Waals force between the lipid molecules and the substrate can perhaps retard the lateral movement of the lipid molecules and thus retain some constraints of the precursor monolayer. Comparing Fig. 4 to Fig. 1, the area per molecule for a bilayer is equivalent to that of a surface monolayer at 47 dyne/cm, the value of A being 40.7 $Å^2$ at this point.

In thermotropic measurements, the membrane vesicles used for differential scanning calorimetry and electron spin resonance experiments are made up of multilayers. Transition temperature measurements by X-ray diffraction and by dilatometry also used bulk lipid/water systems. All of these macroscopic measurements on multilayers give the same value of T_c (= 41.5 °C). In our electron diffraction experiment, we did not detect any inhomogeneous phase transition over the bilayer, the uniform transition temperature being the same as the macroscopic value. For a homogeneous system of dipalmitoylphosphatidylcholine, this agreement is not surprising. However, in a mixed lipid system where phase separation is expected [17, 18] the result may be quite different.

We have constructed a three-dimensional diagram with A, π, and T as three axes, as shown in Fig. 7. The hedged surface in Fig. 7 representing the equilibrium states of the bilayer is roughly parallel to a constant area plane. The dotted surface in Fig. 7 representing the equilibrium states of the monolayer is a curved surface. These surfaces are constructed from their respective projections on the π-T, π-A and T-A planes (Figs 1, 2, 4, 6). The two surfaces intercept roughly along the $\pi = 47$ dyne/cm line. The band of phase transition on the monolayer surface also intercepts the transition line of the bilayer at $\pi = 47$ dyne/cm and $T = 41.5°$, which is also the critical temperature of a monolayer. The coincidence leads us to suggest that in the

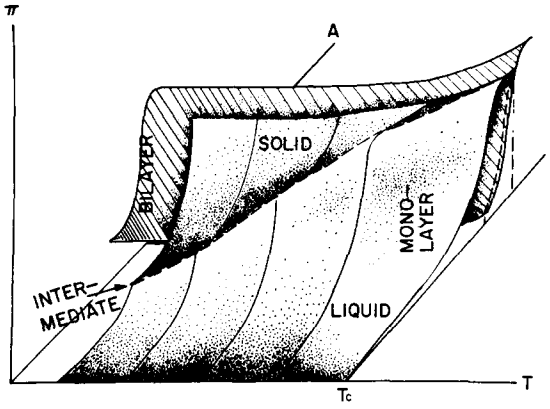

Fig. 7. A three dimensional plot of the area per molecule (A) of bilayer and its precursor monolayer as functions of both temperature (T) and surface pressure (π). The hedged and dotted surfaces represent the equilibrium states of bilayer and of a monolayer respectively. Dashed lines are phase transition lines on the respective surfaces.

neighborhood of the $\pi = 47$ dyne/cm plane the dipalmitoylphosphatidylcholine monolayer has characteristics similar to a bilayer. The isopeizic curves derived from Fig. 1 are plotted in Fig. 6 for comparison. The values are comparable, although the shapes of the curves differ. Since we are considering two different types of membranes here, strict comparison may be questioned. All that can be said at this point is that at $\pi = 47$ dyne/cm, the molecular packing and thermal characteristics of a dipalmitoylphosphatidylcholine monolayer are very similar to that of an unsupported bilayer. This result will be particularly useful in the interpretation of monolayer experiments.

The dried and wet bilayers give approximately the same spacing at room temperature. The thermal expansion effect, however, is quite different at elevated temperatures (Fig. 6). The dried specimen is packed tighter than the wet one and has a lower thermal expansion coefficient. The removal of water molecules seems to increase intermolecular (or interchain) interaction, the molecules being closer together in the dry state. The drying effect in bilayers is very similar to the pressure effect in monolayers, both resulting in transition temperature elevation and in intermolecular spacing reduction. It is obvious that hydration plays an important role in the degree of fluidity of membranes.

In conclusion, it may be said that in a homogeneous dipalmitoylphosphatidylcholine-in-water system, the bulk physical properties are similar to the properties of a single bilayer, the latter in turn corresponding to that of a monolayer at the critical pressure of 47 dyne/cm. These comparisons relate the studies in different systems together, and to the studies of biological membranes [19]. The addition of proteins, cholesterol, cations and other phospholipids is known to alter the characteristics of membranes considerably. The behaviour of biological membranes is thus much more complicated. We intend to use this technique to further our study in this area.

ACKNOWLEDGEMENTS

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