Lipid monolayer states and their relationships to bilayers

(surface pressure/surface potential/membrane/phosphatidylcholine/liposome)

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ABSTRACT Uncommon methods of formation and analysis of lipid monolayers have enabled the recognition of several monolayer states and the identification of that in which molecular organization corresponds closely to that of the bilayer. Monolayers were formed by continuously adding a solution of phospholipid [dimyristoyl phosphatidylcholine in hexane/ethanol, 9:1 (vol/vol)] to the air/water interface of a constant-area trough. This procedure generates unconventional surface pressure (π)–surface concentration (Γ) isotherms, which for liquid-crystalline monolayers consist of straight lines with three prominent intersections, two of which are not apparent in conventional π – A isotherms. The regions of linear change of π are explicable in terms of the area dependence of alkyl chain entropy. The two breaks at lower π delimit states in which both chains lie parallel to the surface. The third occurs at collapse, which corresponds to a true equilibrium for unstressed liposomes. Mechanical and thermodynamic properties of bilayers, particularly phase-transition parameters, correspond closely to those of monolayers with which they are in equilibrium.

Monolayers of amphiphilic substances at air/water interfaces are of interest in a variety of disciplines. In physics and chemistry, the interest is in understanding the origin and magnitude of the molecular interactions of a single layer of complex molecules positioned between two fluids (1, 2). In biology, monolayers have been important since Gorter and Grendel first proposed the bilayer as the foundation of biological membranes (3). Their conclusion was based on an entirely arbitrary compression of a monolayer of lipid extracted from red cells. The critical question of the appropriate compression and, hence, of the relationship between molecular packing in monolayers and that in bilayers remains unanswered.

Using an uncommon constant-area procedure to generate surface pressure (π)–surface concentration (Γ) isotherms of dimyristoyl phosphatidylcholine (Myr₂-PtdCho) monolayers, we have found that the monolayer states of this lipid are characterized by linear regions in the isotherms. There are four such states, and these can be analyzed in terms of their molecular organization. The most condensed state exists in equilibrium with bilayers, and its properties correspond closely to half of a bilayer. This identification is critical for understanding many bilayer properties and is important for membrane reconstitution (4).

MATERIALS AND METHODS

Myr₂-PtdCho, purchased from Avanti Polar Lipids and from Sigma, was dissolved in 9:1 (vol/vol) hexane or pentane/ethanol to a concentration of 0.5 mg/ml. Lipid solution concentrations given are nominal; accurate values were determined by phosphate assay according to a modification of the Bartlett procedure (5). Absolute ethanol was treated with activated charcoal and distilled. Hexane and pentane

were passed through a column of activated alumina. Distilled and deionized water was charcoal-filtered, redeionized, and redistilled. Reagent grade KCl was roasted for an hour or more at 500°C.

Surface tension, γ , was determined from the maximum force exerted on a 0.5-mm-diameter platinum wire as it detached from the liquid surface in a trough on a platform undergoing a 1.5-mm vertical excursion four times per min. The force was measured with a Cahn electrobalance connected to a recorder. Clean water was used for calibration. Teflon or glass troughs (30- to 120-cm² area) with false bottoms for water circulation or for accommodation of a Peltier-effect device were used to control the temperature. The subphase was stirred with a magnetic microbar, and the temperature was measured with a calibrated thermistor.

Records of γ versus Γ were obtained by delivering a solution of lipid at a constant rate of approximately 50 nl/cm² per min from a motor-driven microliter syringe with a Teflon-tipped plunger. The syringe needle was bent nearly 90° and positioned so that the meniscus of the aqueous phase oscillated across the bevel of the needle point. Absence of a significant effect of residual solvent was indicated by two sensitive tests. (i) When delivery was interrupted in the middle of an isotherm, the tension did not change more than 1 dyne/cm (1 dyne/cm = 1×10^{-3} N/m) in the time normally devoted to the entire isotherm. (ii) Repeated addition of solvent to monolayers did not affect surface tension unless enough was added that a lens of solvent appeared, which did not occur under normal conditions of delivery. Under these extreme conditions, γ only changed 1–2 dynes/cm; after the lens evaporated, the tension returned to the original value.

The equilibrium monolayer pressure of liposomes was determined on suspensions of 1.0 mg/ml in 0.01% NaN₃. The trough was covered between measurements, but it was occasionally necessary to add water to replace that lost by evaporation.

Surface potentials were measured with a polonium air electrode and a Ag/AgCl subphase electrode essentially as described (6). The trough area was 7.5 cm². The subphase of approximately 5 ml was continuously stirred at several hundred revolutions per minute with a magnetic microstirrer. Surface tension was monitored simultaneously.

The phase transition of liposome dispersions was detected with a spectrophotometer as described (7). The same thermistor, calibrated against a secondary-standard mercury thermometer, was used for both monolayer and bilayer measurements.

RESULTS

Fig. 1A shows the surface tension (γ) of the air/water interface as a function of the volume of Myr₂-PtdCho solution delivered to the surface at 32°C. The vertical transitions are a result of the periodic contact with and removal of the dipping wire from the surface. The envelope of the upper

ends of the lines describes the surface tension–surface concentration (γ – Γ) relationship. The corresponding surface pressures are presented in Fig. 1B, where the abscissa has been marked in scales for both linear concentration and nonlinear area per molecule. Fig. 1C presents corresponding data obtained at 17°C. At this temperature the phase transition began between 30 and 35 dynes/cm. The data of Fig. 1B and C are presented in Fig. 1D in the more conventional π – A plot in which A is the partial molecular area. Both curves become independent of A at $\gamma = 23$ dynes/cm. The discontinuities at about 140 and 85 Å², so evident in Fig. 1B, are not obvious in the corresponding curve of Fig. 1D. Hence, plotting π against Γ , rather than its inverse ($A = 1/\Gamma$), affords additional information.

The pressure maximum (or tension minimum) seen in the isotherms of Fig. 1 corresponds to the tension of a monolayer in equilibrium with fully hydrated bilayers of large liposomes. This is shown in Fig. 2. At a temperature t above the bilayer phase-transition temperature (t_m), the tension at the air interface of liposome suspensions fell quite rapidly to a plateau value of 23 dynes/cm (49 dynes/cm pressure), which is the same value attained at low areas per molecule in Fig. 1. Thus, both methods of attaining equilibrium (i.e., adding an excess of lipid in solvent and allowing liposome suspensions to stand) generate the same pressure—a further indication of a lack of residual solvent effects in the constant-area

procedure. In fact, solvent actually promoted equilibrium, as shown by the filled diamond of Fig. 2, which represents addition of 7 μ l of hexane (containing no lipid) to the surface of a Myr₂-PtdCho suspension at 26.5°C. The tension immediately fell to the equilibrium value of 23 dynes/cm. When the temperature was reduced below t_m of the bilayer, the equilibration rate diminished by a factor of more than 10².

Monolayers spread to the liposome equilibrium pressure underwent an abrupt phase change at a temperature close to that of the corresponding bilayer transition, according to the temperature dependence of the surface potential of monolayers and the turbidity of bilayer dispersions (Fig. 3). The monolayer transition was only 0.5°C below that of the bilayer transition at 23.5°C. Over the temperature range from 17°C to 32°C, the change in surface potential (ΔV) decreased 32% from 580 to 440 mV, whereas the turbidity decreased 16%. The near-congruence of the curves of Fig. 3 indicates a close correspondence between the organization of lipids in bilayers and those in monolayers at 23 dynes/cm, the liposome equilibrium tension. Although the surface potential measurement was most conveniently done with a monolayer in actual equilibrium with vesicles, essentially the same results were obtained when a monolayer was simply spread to the equilibrium pressure over water.

If the reduction in ΔV (Fig. 3) and the increase in A (Fig. 1) correspond to the crystal–liquid crystal transition of bilayers,

FIG. 1. γ – Γ and π – A relationships for Myr₂-PtdCho. (A) Raw data obtained at 32°C. A hexane/ethanol 9:1 (vol/vol) solution of lipid was delivered to the air/water interface at a constant rate. The surface tension was measured four times per minute by the detachment variation of the Wilhelmy method. (B) The upper extremes of the recorder excursions of A, representing γ , were converted to π and plotted as shown. The arrow indicates the break referred to in the text. (C) As in B but at 17°C. (D) Every fourth point from B and C is replotted as π versus A .

FIG. 2. Myr₂-PtdCho liposomes at $t > t_m$ equilibrate rapidly with the air/water interface to generate a tension corresponding to the minimum γ on γ - Γ isotherms. The surface tension of a suspension of Myr₂-PtdCho liposomes was recorded at different temperatures and as a function of time after aspiration of the surface: 25°C and time in minutes (●), 19°C and time in hours (□), and 17°C and time in hours (○). For the point marked ○, a suspension at 25°C was aspirated, generating a surface with a 70-dyne/cm tension. Addition of a small drop of hexane caused the tension to immediately drop to 23 dynes/cm (●), the equilibrium tension.

the monolayer should exhibit a change in the slope of $d\gamma/dt$ at t_m . Fig. 3 *Inset* shows γ as a function of t for a monolayer initially at equilibrium with liposomes at a temperature just above their t_m (star). Cooling (closed symbols) the monolayer from 25°C to t_m induced a small increase of about 1 dyne/cm, after which γ rose linearly with decreasing t . Upon reheating (open symbols), the same line was obtained as for cooling except for some overcompression between t_m and 29°C. This overcompression, which was not always observed and which partially obscured what otherwise was a sharp change in slope at a tension of 23 dynes/cm and a temperature of 24°C, was metastable and decayed in a few minutes. The value of $d\gamma/dt$ at $t < t_m$ was 2.3 dynes/cm per degree Celsius, which agrees with that for bilayers (see the *Discussion*).

DISCUSSION

Advantages of the Constant-Area Method. Our analysis of phospholipid monolayers was facilitated by three advantages that the constant-area method provides relative to the common compression procedure. First, when coupled with a constant delivery of lipid, the constant-area method directly generates the π - Γ relationship. Second, collapse occurs sharply at the equilibrium pressure of hydrated lipid. Hendriks and Ter-Minassian-Saraga (8) have previously called attention to the latter characteristic and also have demonstrated that comparable π - A results are obtained by the two techniques. The third advantage is speed and simplicity. Introduced by Alexander and Teorell in 1939 (9), the constant-area method has seldom been used, apparently initially because of concern about retention of solvent. This has since been shown to be of little consequence (8, 10), a conclusion supported by our results.

π - Γ Isotherms Generated by the Constant-Area Method Reveal New Information on Monolayer States. The π - Γ plot, in contrast to the π - A plot, consists of segments of straight lines, which imply the existence of several different phases, the equation of state for each segment having the form $\pi = B/A +$

FIG. 3. Monolayers at the equilibrium pressure of liposomes undergo phase transitions that correspond closely to bilayer transitions. One microliter of a solution of Myr₂-PtdCho (10 mg/ml) in pentane/ethanol, 9:1 (vol/vol), was added to the surface of a dispersion of Myr₂-PtdCho (0.5 mg/ml) in 0.1 M KCl, which had been cycled several times through the phase transition and cooled to about 17°C. The temperature was raised about 1°C/min, and the surface potential was recorded (□). Optical density of a suspension of Myr₂-PtdCho was measured as a function of temperature (○). (*Inset*) The $d\pi/dt$ of monolayers at the liposome π exhibits a discontinuity at the bilayer t_m . A suspension of Myr₂-PtdCho liposomes was allowed to equilibrate with its air interface at 26°C (see star). The temperature was then lowered (●) and subsequently raised (□) at a rate (2°C/min) too rapid for significant equilibration between liposomes and the air interface. Thus, the points represent temperature dependence of the tension of the monolayer that was at equilibrium with liposomes at just above their t_m .

C, where B and C are constants. That such behavior is due to a property of the monolayer and is not a consequence of the procedure by which it was generated is indicated by the fact that π - A compression isotherms for Myr₂-PtdCho obtained in other laboratories (11–13), when replotted as π - Γ , also exhibit linear relationships in which a break occurs in the region of 10–15 dynes/cm. Moreover, examination of literature data on polar lipids reveals this behavior to be quite common except when the acyl chains are highly heterogeneous.

The essential features of the isotherms are shown in Fig. 1B. The lowest pressure phase is terminated by an abrupt rise in pressure occurring at an area that is slightly less than that of two myristic acid molecules recumbent on the surface. This orientation follows from the sizable (40–45 ergs/cm²; 1 erg/cm² = 1×10^{-3} J/m²) energy of adhesion of hydrocarbon to water; a Boltzmann distribution calculation shows that fewer than 10% of the chains would have as many as three carbon atoms extending into the air. Thus, this region consists of islands of molecules, one methyl group in thickness, associating laterally and, except at very high areas, exhibiting a two-dimensional vapor pressure. (Note, this vapor pressure will differ from that of a vertically oriented phase, which usually is not experimentally accessible.) The next region extends from about 140 Å² to about 80 Å² (arrow in Fig. 1B). Since the latter area corresponds to the area of the side of a single C₁₄ chain, the molecules evidently roll over from positions where both chains are in contact with water to those in which one chain contacts water and the other is extruded vertically upwards to form, at the limit of this phase, a double layer of horizontally oriented alkyl chains. The third region begins at about 85 Å², at which point any further increase in surface concentration requires that the molecules

reorient from a horizontal to a vertical position. This region is terminated by the onset of the last phase, which occurs where the pressure reaches a constant maximum value, signifying equilibrium with vesicles in the subphase.

Linear π - Γ Relationships Are Consistent with a Surface-Pressure-Dependent Alkyl-Chain Entropy. An explanation of the observed linear π - Γ relationship, which appears to have general applicability, attributes surface pressure to the entropy that each alkyl chain gains when the monolayer area increases. We begin with $\pi = -(\partial G/\partial \mathcal{A})_{T,P} = -(\partial H/\partial \mathcal{A})_{T,P} + T(\partial S/\partial \mathcal{A})_{T,P}$. To evaluate the first term on the right, we note that as a monolayer expands, the polar groups separate, and the alkyl chains are exposed to water. The net result is the replacement of an air/water interface with oil/water and oil/air interfaces. The surface and interfacial energies of decane and water are available from $d\gamma/dt$ (14). For this typical oil/water case, ΔE is almost zero (2 ergs/cm²), and since $\Delta E \equiv \Delta H$, $\partial H/\partial \mathcal{A} \approx 0$. The expression for π is thus dominated by the area dependence of the entropy. This is obtained from $S = mk \ln W$, where k is Boltzmann's constant, m is the number of molecules in the area $\mathcal{A}$, and W is the number of configurations available to the unit whose degrees of freedom are constrained by increasing pressure—here, an alkyl chain. We assume that the monolayer behaves like a normal liquid so that thickness and area are inversely related—i.e., there is essentially no void volume. The number of configurations available to an alkane of n carbon atoms is related to the partial molecular area of a chain by $W = (gA)^n$ where g is a proportionality constant and A represents molecular area. For a molecule with two identical chains, $W = (gA/2)^n$. This may be understood by projecting each methylene group onto a lattice in the plane of the interface. At a given area, there will be, say, p such positions. If the area is increased by a factor of, say, f , the number of positions increases to pf . (Although the occupant of each position changes with time, all positions will be occupied at any given time because the average inclination of the chains increases in accordance with the area increase.) Each carbon atom is constrained by C—C bonds to sample new positions immediately adjacent to old positions, which constraint nevertheless permits nearly a 6-fold increase in area and exceeds the range we need to consider. $A = \mathcal{A}/m$, so $mk \partial \ln(gA/2)^n / \partial \mathcal{A} = mnk/A$, and it follows that $\pi = nkT$. For Myr₂-PtdCho, n should be 14. Thus, this simple treatment predicts a slope in the high-pressure region of the π - Γ curve that approaches 14 kT , quite close to our experimental value of 13 kT (Fig. 1B). Fortunately, g cancels out, for otherwise its calculation would be quite involved (1, 15). What is important, therefore, is not the detailed motions of the chains but that, because the chain ends are tied to the water surface, the entropy of the monolayer becomes a function of the molecular area. Were the end of the molecule not restricted to the interface, the monolayer would simply behave like a bulk liquid, and its entropy would be independent of the surface area. The low surface pressures of molecules such as triglycerides and cholesterol esters can be ascribed to a weak anchoring of their ester functions.

This analysis of surface pressure appears to be consistent with data other than our own. The values of $d\pi/d\Gamma$ that we have measured from literature data are generally close to the predicted values—e.g., compounds with di-C₁₀ (16) and di-C₁₆ chains (ref. 17; 38°C) yield slopes of 10 and 16 kT , respectively.

The slope of π versus Γ in the low-pressure region is 6 kT , or about half that of the high-pressure region. If this region corresponds to the transition from a single to a double layer of horizontal chains, then this change in slope is expected, for until the chains begin to orient vertically, they compete with each other and thus do not behave independently. Finally, at large areas W becomes proportional to the first power of area, and the familiar perfect gas law is generated.

Attempts to fit the entire π - A isotherm with a single function should be regarded cautiously. Langmuir's equation (ref. 18; with 3 kT in place of kT), for example, fits the data of Fig. 1D (32°C) very well, yet his approach clearly does not anticipate the change in slope seen in the π - Γ relationship.

The Equilibrium Pressure of Fully Hydrated Liquid-Crystalline Lipid (Liposomes) Is the Maximum Surface Pressure Obtainable by the Constant-Area Method. The collapse pressure obtained by the compression method often depends on experimental conditions, in contrast to the constant-area method, where repeated application of small drops of solvent lowers the activation energy for escape of lipid from the monolayer and prevents the tension from falling below the equilibrium value of a liposome suspension (23 dynes/cm for Myr₂-PtdCho). An earlier application of the constant-area method to stearic acid showed that collapse occurred at the equilibrium pressure (8). The limiting tension remains the equilibrium tension of liquid-crystalline liposomes even at temperatures below t_m because the solvent effectively melts the monolayer at the point of addition despite the gel nature of the existing monolayer. In principle, the equilibrium tension at $t < t_m$ can be obtained by allowing gel-phase liposomes to equilibrate with the air/water interface, but this is a slow process (>2 days for the 17°C and 19°C Myr₂-PtdCho monolayers of Fig. 3), and extrapolation to an apparent asymptote is problematic.

There is some controversy regarding equilibrium pressures of monolayers below the lipid phase-transition temperature (13, 19, 20). In our experience, the rate of equilibration is strongly influenced by the purity of the lipid and the method of sample preparation (see also ref. 21). Thus, high sample purity and preparation methods that minimize defects in bilayer organization may greatly lengthen equilibration times. Such a "kinetic trap" could explain some variation reported in the literature (22). Surface potential measurements show that our procedure generates monolayers, even up to 32°C (Fig. 3), although bilayers can form at the air/water interface at lower temperatures (23).

The Organization of Molecules in Liposomes Corresponds Closely to that in Monolayers in Equilibrium with Those Liposomes. *Phase-transition temperature.* The data of Fig. 3 suggest a near-equivalence of the lateral interactions of bilayers and equilibrium monolayers. The half-degree difference in transition temperature (Fig. 3) represents 1 dyne/cm (Fig. 3 *Inset*). Part of this small difference may be real, but part also may be due to van der Waals forces that extend across bilayers but not monolayers. Importantly, the foregoing considerations imply a lack of significant coupling across the bilayer of phosphatidylcholines with alkyl chains of similar lengths, as is generally accepted (1). This need not be so in special cases (24), however.

The surface energy of the water/lipid interface. Following the approach of Langmuir (18), we take the monolayer tension to be the sum of tensions at the upper and lower interfaces, an oil/air tension and a polar surface/water tension, respectively. Myr₂-PtdCho has alkyl chains of 14 carbons, but at the tensions under consideration, the upper surface consists essentially of methyl groups. Examination of the surface tensions of isometric alkanes (25) reveals that a hypothetical all-methyl hydrocarbon would have a surface tension 3 dynes/cm lower than the corresponding normal alkane. Applying this correction to the surface tension of tetradecane (26 dynes/cm) yields 23 dynes/cm. Since 23 dynes/cm is the tension of a monolayer in equilibrium with large liposomes, the tension at the lower interface must be essentially zero, as is the measured tension of planar phosphatidylcholine bilayers (26). Therefore, the molecular packing and lateral pressures of the equilibrium monolayer and the bilayer system are virtually the same. (A difference in tension of as much as ± 2 dynes would lead to a difference in area of

not more than $\pm 4\%$.) Our conclusion agrees with the experimental study of Hui *et al.* on monolayers of Myr₂-PtdCho (27), that of Tancrede *et al.* on the monolayer pressure requirements for stable bilayer formation (28), and the theoretical analyses of Nagle (1) and of Gruen and Wolfe (29).

Area per phospholipid molecule. An area per molecule of 53 Å² at 32°C (Fig. 1 B and D) is in general agreement with areas at collapse obtained with conventional techniques (11, 23, 30). More to the point, however, is the relationship between the monolayer and the bilayer. X-ray diffraction on liquid-crystalline bilayers yields larger areas varying from 55 Å² (24) to 65 Å² (31, 32); however, the indirect methods used (33) are extremely sensitive to the concentration of water in the bilayer, trace impurities, and uncertainties in the partial molar volume of water and lipid, all of which may contribute to variation in the published data (34). An area of 53 Å² is in agreement with the directly measured area change in bilayers at the phase-transition temperature, given an area per molecule of 40.5 Å² in the crystalline phase (see the next section). The latter area corresponds to twice the accepted cross-sectional area of single acyl chains. Areas larger than 65 Å² would require the average C—C bond to be more parallel than perpendicular to the membrane plane.

The area change at the monolayer phase transition. A 31% change in monolayer area occurs at the equilibrium (23 dynes/cm) tension when the temperature is raised from 17°C (40.5 Å²) to 32°C (53 Å²) (Fig. 1C). A 31% change also occurs when Myr₂-PtdCho bilayers undergo conversion from the rippled P_{B'} phase to the liquid-crystalline L_α phase (D. Needham and E. A. Evans, personal communication). (The Needham and Evans data, obtained by pipette aspiration of giant vesicles, match our monolayer data of Fig. 3 to within the width of the symbols over the entire temperature range.) The P_{B'} phase is characterized by a rippled bilayer below t_m with tilted chains (32). The tilt and ripple angles must be the same; otherwise, the bilayer structure would change at each bend of the ripple. It then follows that the chains must be perpendicular to the global plane of the membrane. The ripple has an amplitude of 30 Å and a period of 120 Å (35). These dimensions are almost exactly those necessary to generate a symmetrical ripple wherein the chains are perpendicular to the global plane of the membrane and are displaced vertically by one methylene group and are also in good agreement with the 10–12% area dilation that occurs upon stretching out the ripples of Myr₂-PtdCho vesicles (D. Needham and E. A. Evans, personal communication). Thus, the area per molecule below t_m of 40.5 Å² that we have found is as expected for the P_{B'} phase—namely, that of two close-packed alkyl chains.

Mechanical properties. The compressibility modulus, K , of Myr₂-PtdCho vesicles at 29°C is 75 ± 5 dynes/cm per uncoupled monolayer (36). $K = 96$ dynes/cm is obtained for monolayers at 32°C by extrapolation to the equilibrium pressure of the 13 kT slope of the isotherm. The actual value at the equilibrium pressure is 70 dynes/cm, although the slope in this region is somewhat variable. K in the coexistence region is low, as expected, being 21 dynes/cm at 53 Å² and in reasonable agreement with the 10–16 dynes/cm value for half a bilayer (D. Needham and E. A. Evans, personal communication). K of solid-phase bilayers depends on their history. At 16°C it is about 31 dynes/cm per monolayer before and about 135 dynes/cm after the ripples have been pulled out (D. Needham and E. A. Evans, personal communication). We found 60 dynes/cm. From Fig. 3 *Inset*, $d\pi/dt$ is 2.3 dynes/cm per degree Celsius, in good agreement with the 2.75 dynes/cm per degree Celsius for half a Myr₂-PtdCho bilayer (36). By the Clausius–Clapyron equation, the latter agrees with calorimetry values (36).

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