AN ALTERNATIVE VIEW OF PHOSPHOLIPID PHASE BEHAVIOR AT THE AIR-WATER INTERFACE

MICROSCOPE AND FILM BALANCE STUDIES

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ABSTRACT Pure-lipid films at the air-water interface have surface-pressure vs. area isotherms that are often interpreted as involving first-order phase transitions from a condensed region to a liquid-expanded region. Two phases are presumed to coexist in the intermediate part of the isotherm. We constructed a film balance that could be placed on the stage of an epifluorescence microscope. A dipalmitoyl phosphatidylcholine film containing a low concentration of a fluorescent lipid probe showed an inhomogeneous fluorescence distribution in the so-called liquid-expanded region of the isotherm. Only the intermediate and condensed regions could be prepared so as to be optically homogeneous below 25°C. We investigated membrane flow and lateral lipid diffusion in the membrane on the trough. The isotherms and isochores were measured. The results require, at least, a modified description of the monolayer structure in various regions of the isotherms. The solid-condensed region corresponds to a gel phase of the lipids where there is no flow in the membrane, lateral diffusion is low, the compressibility is low, and the membrane is optically homogeneous. The "liquid-condensed/liquid-expanded" region appears to be a homogeneous membrane where lateral diffusion and membrane flow are both rapid. This is a region of high compressibility. The "liquid-expanded" region is not homogeneous as seen under the microscope, and the flow of the surface layer can be very fast.

INTRODUCTION

Films of lipids at the air-water interface are used with increasing frequency as model systems to study physical properties of membranes and the interactions of lipids with other components of biological membranes (Cadenhead et al., 1976; Phillips, 1972; Albrecht et al., 1978; Jähnig et al., 1979; Schindler and Quast, 1980; Klappauf and Schubert, 1978). Monolayers of fatty acids and lipids are usually studied at the air-water interface using a film balance (Bücher et al., 1969; Fromherz, 1971; Kuhn et al., 1972). To describe the different regions in a pressure-area diagram, we will use the nomenclature introduced by Cadenhead et al. (1980). The solid-condensed (SC)[1] region at low areas per molecule is followed by the liquid-condensed (LC) region at larger areas per molecule. At large areas, the monolayer is in

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[1] *Abbreviations used in this paper:* DPPC, dipalmitoyl phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; LC, liquid condensed; LE, liquid expanded; NBD-PE, *N*-4-nitro-benzo-2-oxa-1,3-diazole-L-α-dimyristoylphosphatidylethanolamine; SC, solid condensed.

a "liquid-expanded" (LE) state. The intermediate region between the LC and LE is the LC/LE region.

The parameters that can be varied are the pressure of the surroundings, the surface pressure, π, the average molecular area, A, and the temperature, T. Usually, π vs. A isotherms are measured (Phillips and Chapman, 1968; Seelig et al., 1980); in some instances there have been measurements of the temperature vs. area at constant π (Albrecht et al., 1978), or T vs. π at constant A (Purdon et al., 1976). The principles of thermodynamics at the surface are described by Adamson (1976), Gaines (1966) and Dash and Ruvalds (1980).

Because one can measure so many physical properties of lipid monolayers at the air-water interface, there has been much interest in relating these properties to the physical properties of lipid bilayers which are present in biological membranes. One of the pronounced thermodynamic properties of many pure phospholipid bilayers is the endothermic chain-melting transition, sometimes called the Chapman transition. At temperatures below the Chapman transition temperature, θ_C, for a given phospholipid, the fatty acid acyl chains are essentially fully extended, having the all-*trans* conformation. At temperatures above θ_C, the chains are signficantly disordered. Below θ_C, the bilayer is "solid" (crystalline) and the lateral diffusion of fluorescent lipid probes is low ($D \lesssim 10^{-10}$ cm^2/s). Above θ_C, the bilayer is "fluid," the chains have a number of *gauche* configurations, and the lateral diffusion of fluorescent lipid probes is rapid ($D \simeq 10^{-7}$–10^{-8} cm^2/s). The large endothermic heat absorption at the transition temperature θ_C observed for a number of phospholipids leaves little doubt that this transition is first-order, at least in multilamellar liposomes. Throughout this paper, we assume that the Chapman transition is a first-order transition.

There has been great interest in possible relationships between the Chapman transition in bilayers and the π-A isotherms in lipid monolayers at the air-water interface. A strictly first-order transition in a lipid monolayer should appear as a horizontal line (constant π) in a π vs. A isotherm. No measurement has yet shown this behavior for phospholipids at the air-water interface, although the LC/LE region has isotherms that are nearly horizontal at lower temperatures for some phospholipids. One of the more recent interpretations of the LC/LE region is that it represents a "diffuse first-order" transition, a transition that is broadened because the cooperative units involved are finite (e.g., ~100 molecules) (Cadenhead et al., 1980; Albrecht et al., 1978). However, other interpretations of the phospholipid π-A isotherms have been made, and the subject remains controversial. For a review and discussion of theories of phase transitions in monolayers and bilayers, see Nagle (1980).

The present work was originally motivated by a desire to transfer lipid monolayers to glass slides for studies of cellular immune response to specific molecules incorporated into the monolayers. We discovered that when fluorescent lipid probes were included in the monolayers, the monolayers on the glass slides sometimes showed various types of inhomogeneities and that corresponding inhomogeneities could also be observed on the parent lipid film on the air-water interface by epifluorescence microscopy (von Tscharner and McConnell [1981]). We therefore decided to measure π-A isotherms (as well as isochores) to compare our monolayers with the monolayers extensively studied in other laboratories. Our measured π-A isotherms are in good agreement with those measured in other laboratories, but under some conditions, the fluorescence of these films is in obvious disagreement with some published

interpretations of the monolayer data. We therefore give a brief discussion of the relevance of our data to the interpretation of the π-A isotherms for lipid monolayers.

MATERIALS AND METHODS

L-α-dipalmitoyl phosphatidylcholine (DPPC) and L-α-dimyristoyl phosphatidylcholine (DMPC) were purchased from Sigma Chemical Co. (St. Louis, Mo.; 98% pure) and used without further purification. Some measurements were made with recrystallized DPPC, but no difference was observed.

N-4-nitrobenzo-2-oxa-1,3-diazole-L-α-dimyristoyl phosphatidylethanolamine (NBD-PE) was purchased from Avanti Biochemicals, Inc. (Birmingham, Ala.) and was used without further purification. The water was prepared using a Milli Q Water Purification System (Millipore Corp., Bedford, Mass.).

After filling the trough with water, the Wilhelmy balance showed no increasing surface pressure with time. No cleaning of the surface was needed. To test the purity of the solvent, we applied three times the amount of solvent that we usually used for spreading, and compressed it to the minimal area possible. As no pressure increase was found, the area could then be reduced to 15% of the initial area without increasing the surface pressure. The lipid was dissolved in a hexane-ethanol mixture (9:1) and was applied to the surface with a Hamilton syringe (Hamilton Co., Reno, Nev.). The surface pressure was measured by the Wilhelmy method (Bücher, 1969). We used a piece of Millipore filter (GS, 0.22 μm) ~2.5 cm wide as a Wilhelmy plate; this was attached to a Roller Smith torsion balance (Biolar Corp., N. Grafton, Mass.), and this proved to give very reproducible measurements. Less than 0.5 dyn/cm difference in the baseline pressure was observed between the beginning and end of an experiment, which sometimes lasted >24 h. The amount of water in the filter was very small and the filter was rigid enough to remain flat. The system was reproducible to within 0.1 dyn/cm. For a comparison of different methods, see Boucher et al. (1967). The absolute accuracy was mainly determined by the Wilhelmy plate, and we estimate it to be better than 5%.

We used a Teflon ribbon (Chemfluor LAB-TAPE; Chemplast Inc., Wayne, N. J.) to adjust the area, which we pulled over the four screws (hydrophobic) that formed the corners of an aluminum frame. The frame was made of four rods of equal length and had the shape of a rhombus. As we changed the height of the rhombus, the area covered could be calculated as base times height. Such a rhombus can be constructed with any degree of sophistication required. A threaded rod across one diagonal can be used to adjust the height, or simple models can be moved by hand. The advantages of such a rhombus are that it can be used standing on the four screws in any trough, it can hang from a supporting panel (cover), and it is very easy to clean and to insert. One can use the inside or the outside of the rhombus for the application of the films. We usually used the inside because larger relative changes in surface area can be achieved. The whole rhombus can be moved around on the trough to bring the membrane into another position within the trough. The size is limited by the elasticity of the ribbon used, and the dimensions must be chosen so that the Wilhelmy plate does not come too close to an edge. There is no possibility for the lipids to leak out, and the circumference is not changed when the area is altered; therefore, errors from edge effects are minimal. There is no need to fill the trough to the rim; we always used a height such that the accuracy in the determination of the area was better than 0.5%. Our first trough was made of Teflon-coated aluminum and had the dimensions 35.6 × 24.1 cm. The base of the rhombus we used was 15.6 cm, and the depth of the water was 1.12 cm. The trough was placed in a circulating water bath or on a heating plate and was covered by a Plexiglass hood. The whole apparatus was enclosed in a heated cabinet. The second trough had a depth of only 2 mm, a length of 20 cm, and a width of 15 cm. It was milled from an aluminum plate. The rhombus had a base of 12 cm. We placed this trough on the thermostat-controlled stage of a Zeiss photomicroscope III (Carl Zeiss, Inc., New York; magnification, 800 or 200). 1 mol/100 mol of the fluorescent label NBD-PE was included in the lipid so we could observe the monolayer using this same epifluorescence microscope. The temperature of the monolayer was measured by a thermocouple that was inserted through the monolayer and was <1 mm below the surface. The temperature homogeneity was better than 0.2°C over the whole covered

area. A mark on the thermocouple was used to determine the water level, which was readjusted from time to time.

The slopes of plots of π vs. $\ln A$ are equal to $-\kappa^{-1}$, where κ is the compressibility. By plotting π vs. $\ln A$, one can separate the determination of the area per molecule, which can be made at a specific pressure, e.g., 40 dyn/cm, and the determination of κ^{-1}. Graphs of π vs. $\ln A$ can be shifted along the $\ln A$ axes for comparison with other measurements that have often been made with imprecisely known amounts of lipid. Comparison with results from other laboratories should thus be easier.

RESULTS

We applied 30 μl of 1.5 mM DPPC onto a clean, fully expanded trough. After 30 min, we slowly compressed it to $\pi > 40$ dyn/cm (Fig. 1, *upper curve*). Then the surface pressure π dropped at a rate $\ll 1$ dyn/min. We had to wait ~2 h to reach equilibrium, which was ~8 ± 2 dyn/cm below the starting pressure. This relaxation has been observed before and cannot be expressed by a single exponential (Tabak et al., 1977). We recompressed to the starting value, which was >40 dyn/cm. At 40 dyn/cm, we usually found an area per molecule of 40 ± 2 Å^2. These areas are ~5 ± 2% smaller than the ones measured immediately after compression. We observed the lower curve shown in Fig. 1 upon expanding the area. The shapes of the curves

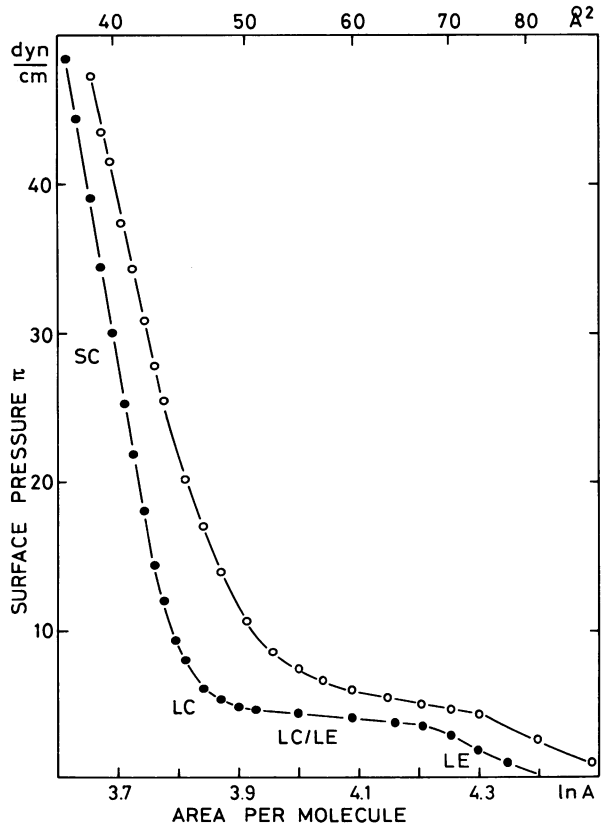

FIGURE 1 Pressure area isotherms of DPPC measured by decreasing the area (○) and by increasing the area (●) after the monlayer was left at high pressure for 2 h. The temperature was 20.4°C.

were not much altered if we included 1% NBD-PE in the lipid sample. (For other methods of preparing lipid monolayers and a discussion of their metastability, see Horn and Gershfeld, 1977.)

As shown in Fig. 1, there is some hysteresis in the π vs. A curves. There was a difference that depended on whether one observed the hysteresis of the whole π-A curve or only local regions. After the initial compression and equilibration described above, we tested the reversibility of the different regions at a temperature between 20 and 25°C. The areas measured in the SC region by decreasing the pressure are <1% apart from those measured by increasing the pressure. The monolayer in the SC region shows no relaxation or hysteresis. (Note that in this paper the terms SC, LC, LC/LE, and LE are defined by the regions of the lower curve in Fig. 1. For a detailed discussion of the terminology, see Cadenhead, 1980.) When the monolayer was allowed to expand to an area corresponding to the LC state, there was a slow increase in pressure with time. The pressures first measured lie on the extrapolated line from the SC region to larger areas. The final pressure was a few dynes higher. The initial rate of the increase in pressure was ~1 dyn in 10 min, and it leveled off after 1 h. For most π-A measurements, we waited only a few minutes for each point. The monolayer in the LC state could be recompressed to the SC state but we had to wait at high pressures as described for the initial procedure. The π vs. A diagram is not reversible in the LC region. On the other hand, the LE/LC region showed no hysteresis. After an increase and a decrease of area in the LC/LE region, the pressure showed a <0.4-dyn difference.

If we increased the area still further to reach the LE region, no reversal was possible. If we expanded the monolayer to π = 0.5 dyn/cm and recompressed immediately, we found a π-A curve between the initial compression and the final decompression curve. The pressure in the LE state increased with time and reached the initial starting pressure if we waited long enough (40 min).

We prepared an identical monolayer under the microscope and left it in the LE state for only 5 min before it was compressed to the area known to correspond to the condensed phase. The membrane looked very patchy. These patches were small in the condensed region and gradually expanded as the total area of the membrane was increased. The patchy area consisted of a mixture of brighter and darker areas. Each area in itself looked homogeneous and was fluorescent. If we left the lipids at 23°C for 30 min before we compressed the membrane, then the condensed phase appeared to be optically homogeneous. (If not otherwise stated, we used only optically homogeneous monolayers for recording monolayer properties.) Overcompression of a monolayer resulted in long, sharp ruptures of the membrane. These ruptures are very bright, they can be ≥1 cm, and are so far apart from each other that it is often hard to find them with a magnification of 200. The absorption transition dipole giving rise to the fluorescence of NBD-PE in lipid bilayers is known to be oriented preferentially perpendicular to the bilayer surface (Smith et al., 1981). These bright ruptures may have the structures described by Kézdy (see Fig. 5 *A* in Kézdy, 1972). As we expanded an optically homogeneous membrane, we observed the movements of small imperfections or dust particles. In the SC region, almost no movement of the particles was observed. By blowing on the surface, an elastic movement of the particles was observed, but they then returned close to their original positions.

Photobleaching was used to estimate the magnitude of the lateral diffusion coefficient of

the fluorescent lipid probes. The image of the edge of a specially constructed image plane shutter on the microscope could be seen on the membrane as a line formed between a light-exposed, fluorescence photobleached area and an unbleached area, which was shielded by the shutter. Such a bleached pattern could be observed ≤ 1 min after the shutter was removed. The elasticity of the membrane and small vibrations made an accurate diffusion measurement by the pattern photobleaching technique impossible (Smith et al., 1978). Multilamellar lipid membranes that are in the gel phase have lipid diffusion constants, D, $<10^{-10}$ cm^2/s (Rubenstein et al., 1979). They show long-lived bleach patterns similar to our monolayer. In the LC and LC/LE regions, the membrane was also optically homogeneous, but the whole membrane moved slowly across the field of view. (Our microscope was placed on a pneumatically supported optical table, which underwent low-frequency, low-amplitude oscillations. The small tilting of the microscope was presumably responsible for the membrane flow.) The relative distance between particles did not change; their separation was maintained by the membrane. No bleach pattern remained on the surface, indicating rapid lateral diffusion and membrane continuity. When we reached the LE region, the membrane suddenly

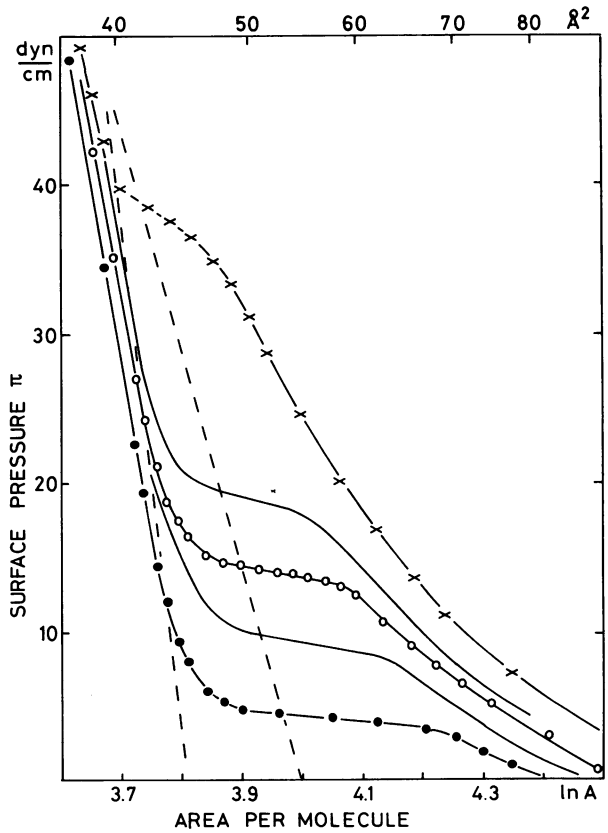

FIGURE 2 Pressure-area isotherms of DPPC. The temperatures for the isotherms are 20.4, 25, 27.5, 30, and 37.5°C. The dotted lines mark the beginning and the end of the liquid-condensed region. The points at the beginning and the end of the LC/LE region have been defined as M1 and M2 by Albrecht et al. (1978). The temperature was 25.5°C.

looked very patchy. It became very hard to focus the microscope because the movement of the monolayer is fast. The bright and dark areas still moved together and there was little observable relative motion. Above 25°C, the same fluidity properties were observed but no inhomogeneities could be resolved. The measurements of π vs. $\ln A$ are shown in Fig. 2. If we plot the $d\pi/d\ln A$, we find the compressibility shown in Fig. 3. Again, we have assigned the different regions according to Cadenhead (1980).

In the SC region, κ was nearly constant. Then as we reached the LC region, a sharp increase in compressibility occurred. The shape of the curve in Fig. 3 in the LC region is very much dependent upon the time in which the system is allowed to relax. The compressibility in the LC/LE region was large and changed only slightly as the area was changed. When the monolayer entered the LE region, κ decreased rapidly and remained relatively constant for larger areas. The resolution of the instrument did not allow us to resolve a more detailed structure within the breaks illustrated in Fig. 3. Determinations of κ by Albrecht et al. (1978) indicate additional, smaller breaks in κ near 70 and 45 Å^2; these were sometimes just barely detected in the present work; they were strongly dependent on the time taken to make a measurement.

The points in a π vs. $\ln A$ plot at which the SC region ends and the LC region starts determine a negative tilted line for the onset of this transition (Fig. 2, leftmost dotted line). That it is negatively tilted allowed us to melt the lipids at constant area. It was critical to start at a proper area within the SC region that had a low enough pressure such that the whole transition could be observed by increasing the temperature (Fig. 4). For an area of 41.7 Å^2/molecule we observed a linear increase in pressure with temperature in the SC and LC regions. $(d\pi/dT)_{SC} = 0.5$ dyn·cm^{-1}·°C^{-1}. The slope $(d\pi/dT)$ of the LC/LE region is linear within the temperature range of the observation at an area of 41.7 Å^2/molecule;

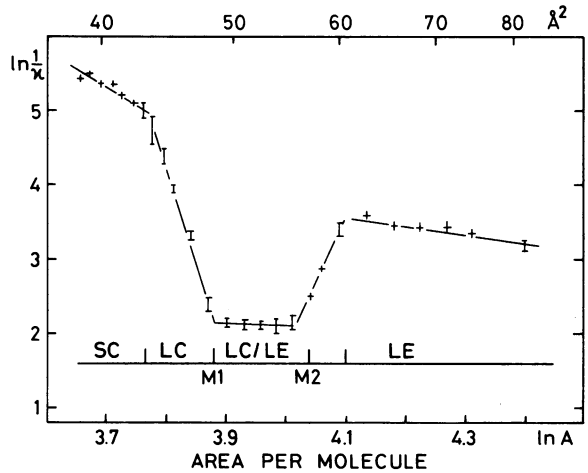

FIGURE 3 The compressibility vs. area graph was derived from measurements of the π vs. $\ln A$ plots (Fig. 2). The derivative was calculated by fitting a number of sets of four consecutive points to a quadratic equation. The slope of the fitted curve was calculated for the two center points. This procedure was continued moving the array of points further by one point. If the two values obtained for each point were different, they were joined them by a line.

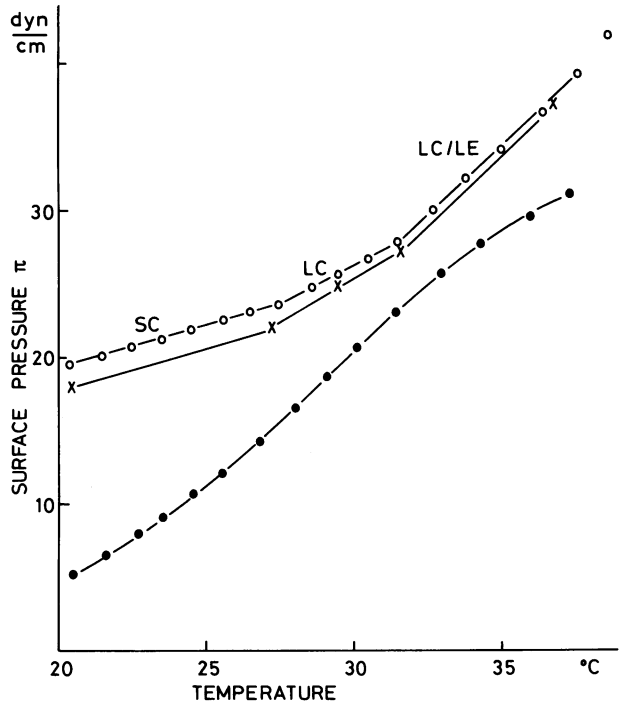

FIGURE 4 Melting of the DPPC monolayer at constant area after decompression of the monolayer. O, increasing the temperature at A = 42 Å^2/molecule; ×, decreasing the temperature at A = 42 Å^2/molecule; ●, increasing the temperature at A = 49 Å^2/molecule. The curves and calculated slopes were not corrected for the change in surface tension of the water. The slopes at 42 Å^2/molecule at increasing temperature are 0.5, 1.0, 1.85 dyn·cm^{-1}·°C^{-1} for the SC, LC, and LC/LE regions, respectively.

$(d\pi/dT)_{LC/LE}$ = 1.86 dyn·cm−1·°C^{-1}, but is not linear, for instance, at 49 Å^2/molecule $1.4 < (d\pi/dT)_{LC/LE} < 1.9$ dyn·cm^{-1}·°C^{-1} (Fig. 4).

DISCUSSION

As indicated in the Introduction, this work was not initiated as a study of phase equilibria in lipid monolayers at the air-water interface. Our purpose was to find conditions under which lipid monolayers containing fluorescent lipid probes could be transferred from an air-water interface to a glass slide with retention of certain physical properties and with homogeneity of fluorescence intensity. As shown in the accompanying paper (von Tscharner and McConnell, 1981), this effort was successful. Even though our film balance was not designed to have optimum sensitivity and stability, our monolayer data are in good accord with results of more sophisticated investigations, and the epifluorescence microscopic observations of these films provide significant new information concerning their physical properties. Our observations and conclusions that bear most significantly on earlier experimental and theoretical work are as follows.

(*a*) At lower temperatures (between 20 and 25°C) the LE monolayer is never homoge-

neous. The fluorescence shows darker and brighter areas. We suspect that the bright areas are lipids in the fluid phase, whereas the darker areas are occupied by lipids that have lost their membrane-like structure and that fill the space between the membrane patches. The relaxation observed in the LE region after decompression of the monolayer reflects a slow disordering process. This disordering process may partly parallel the ordering that occurs at high pressure. (At high enough temperatures, the transition from the LE to the LC/LE regions is less pronounced.) The principal shape of the π vs. $\ln A$ curves are also shown for the inhomogeneous monolayers.

(*b*) On the basis of our results, we have no reason to identify the LC/LE region with two coexisting phases related to the Chapman transition in bilayers, i.e., a transition between a "fluid" lipid and a "solid" lipid. An assignment of this type has been suggested by Cadenhead et al. (1980), and by Albrecht et al. (1978). In the present work, the LC/LE region (cf. Figs. 1 and 3) is optically homogeneous, suggesting the absence of coexisting phases with domains having sizes comparable to or larger than the wavelength of light. If two phases did exist in the LC/LE region, they would both have to be fluid phases; otherwise one would expect a pronounced reduction of the lateral diffusion coefficient of the fluorescent lipid probe NBD-PE or the relative concentration of the presumed LC phase would have to be increased. Our data in the LC/LE region are consistent with two alternative views: (*i*) The LC/LE region is a single homogeneous phase. (*ii*) The LC/LE region is a two-phase region, related to the L_α-$P_{\beta'}$ phase transition in bilayers, except that the $P_{\beta'}$ phase is fluid in monolayers. For a description of these bilayer phases, see Luzzati (1968).

(*c*) The LC region, shown clearly in Fig. 3, does have characteristics of a transition region: between fluid and solid monolayers. At the lower lipid areas of ~44 Å^2, the lateral diffusion of the fluorescent lipid probe was low, whereas at ~48 Å^2 this lateral diffusion was large. The marked change in $\ln(1/\kappa)$ in this region also suggests a phase change. It is easily shown that

$$\ln(1/\kappa) = n(dG/d\pi) - n(d^2G/d\pi^2), \tag{1}$$

so that either a first- or second-order transition can give rise (ideally) to a discontinuous change in $\ln(dG/d\pi)$ or $\ln(d^2G/d\pi^2)$. Here G is the Gibbs surface free energy. The strong hysteresis reported above in this region supports the idea of a first-order transition. The absence of true discontinuties in $\ln\kappa$ in this region is readily understood in terms of the pronounced hysteresis in this LC region.

(*d*) In the SC region, the lateral diffusion coefficient of the lipid probe was very low ($D \lesssim 10^{-10}$ cm^2/s) and thus similar to the diffusion of this probe in "solid" lipid bilayers. After equilibration, the SC region showed no hysteresis if the variables and A remain within this region. Thus, the SC region appears to have the properties of a single phase, analagous to the $L_{\beta'}$ (or perhaps $P_{\beta'}$) phase of phospholipid bilayers.

In conclusion, we note that the simplest single interpretation of our data is that (*a*) the LE region is a two-phase region, part of which is membrane-like and the other part not, (*b*) the LC/LE region is a single, homogeneous phase, (*c*) the LC region is a two-phase region roughly contained within the area represented by the dashed lines in Fig. 2, and (*d*) the SC region is a solid, single-phase region. Further studies of monolayer membranes using epifluorescence microscopy are likely to be rewarding, but the marked hysteresis shown by

these systems must somehow be understood and/or controlled before completely satisfactory studies can be carried out.

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