Epifluorescence Microscopic Studies of Monolayers Containing Mixtures of Dioleoyl- and Dipalmitoylphosphatidylcholines

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ABSTRACT Monolayers of dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), and some mixtures of these lipids were investigated using an epifluorescence microscopic surface balance. Monolayers were visualized at 23 ± 1°C through the fluorescence of 1 mol% of two different fluorescent probes, 1-palmitoyl-2-{12-[(7-nitro-2–1,3-benzoxadizole-4-yl)amino]dodecanoyl}phosphatidylcholine (NBD-PC), which partitions into the liquid expanded (LE) or disordered lipid phase and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO-C_{18}), which preferentially associates with the liquid condensed (LC) phase or lipid with ordered chains. LC domains were observed in pure DPPC monolayers at relatively low surface pressures (π), and these domains grew with increasing surface pressure. Only liquid expanded phase was observed in pure DOPC monolayers up to the point of monolayer collapse. In monolayers containing 29:70:1, 49:50:1, and 69:30:1 (mol/mol/mol) of DPPC:DOPC:probe the domains of LC phase were smaller than those seen in DPPC monolayers at equivalent surface pressures. Quantitative analysis of the visual fields shown by the mixed monolayers showed a distribution of sizes of condensed domains at any given π. At π = 30 mN m^{-1}, liquid-expanded, or fluid, regions occupied more than 70% of the total monolayer area in all three mixtures studied, whereas DPPC monolayers were more than 75% condensed or solid at that pressure. For monolayers of DPPC:DOPC:NBD-PC 49:50:1 and 69:30:1 the average domain size and the percentage of the total area covered with LC, or rigid, areas increased to a maximum at π around 35 mN m^{-1} followed by a decrease at higher π. Repetitive compression and expansion of the monolayers containing DPPC:DOPC:NBD-PC 49:50:1 at an initial rate of 3.2 $Å^2$ $molecule^{-1}$ s^{-1} produced monolayers with visual properties consistent with there being a preferential exclusion of the unsaturated lipid from the monolayer.

INTRODUCTION

Monolayers of lipids at the air-water interface have long served as models for biological membranes. Also it is generally accepted that lipid monolayers are responsible for the lowering of surface tension in the lung by pulmonary surfactant. A comprehension of the two-dimensional distribution of lipids in mixtures in monolayers is fundamental to understanding of pulmonary surfactant function, and is highly relevant to our perception of two-dimensional segregation of lipids in bilayers. In this study we employ the technique of epifluorescence microscopy of monolayers to study a set of simple mixtures which are relevant to our perception of how both biological system could be organized.

Pulmonary surfactant spontaneously spreads into the air-water interface to form a monolayer in vitro (King and Clements, 1972; Wildeboer-venema, 1978; Bangham et al., 1979; Hildebran et al., 1979). It is assumed that in the lungs the monolayer is responsible for maintaining low surface tension at the air-alveolar fluid interface that facilitates lung function and prevents the lungs from collapse during expiration (King and Clements, 1972; Schürch et al., 1976; Wildeboer-venema, 1978). When compressed in monolayers, DPPC, the major component of the pulmonary surfactant, can withstand high surface pressures of about 70 mN m^{-1}, corresponding to very low surface tensions. Pulmonary surfactant contains some unsaturated lipid species which do not withstand such high π; these have been suggested to play other roles such as facilitating adsorption and spreading of material into the interface (Bangham et al., 1979; Hildebran et al., 1979; Notter et al., 1980). DPPC can neither rapidly adsorb from a aqueous subphase nor respread from collapsed phases of monolayers after compression.

Monolayers of lipids have long served as models for biological membranes. In bilayers, DPPC shows a chain melting phase transition (T_c) near 41°C (Bashford et al., 1976; Davis et al., 1980). In PC monolayers at temperatures below T_c, closely packed molecules can sustain high π, or produce very low surface tensions, at the air-water interface (Hawco et al., 1981). Monolayers of unsaturated lipids such as DOPC (with T_c below the measurement temperature) do not withstand very high π, and they collapse at a surface pressure near 50 mN m^{-1} (Notter et al., 1980). It has been suggested that the unsaturated components are preferentially excluded during compression of a pulmonary surfactant monolayer to enrich the monolayer with DPPC (King and Clements, 1972; Bangham et al., 1979; Notter et al., 1980; Hawco et al., 1981a, 1981b; Egberts et al., 1989).

Monolayers of saturated phosphatidylcholines containing chain lengths of more than 10 carbons exhibit surface pressure-induced phase transitions (Blume, 1979; Notter

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Abbreviations used: π, surface pressure; DOPC, 1,2,-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2,-dipalmitoyl-*sn*-glycero-3-phosphocholine; DiO-C_{18}, 3,3′-dioctadecyloxacarbocyanine perchlorate; LE, liquid expanded; LC, liquid condensed; NBD-PC, 1-palmitoyl-2-{12-[(7-nitro-2–1,3-benzoxadizole-4-yl)amino]dodecanoyl}phosphatidylcholine; T_c, gel to liquid crystalline transition temperature.

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et al., 1980). The phase transitions of DPPC have been studied extensively in the last two decades by different techniques, which, recently have included epifluorescence microscopy (Gaub et al., 1986; Flörsheimer and Möhwald, 1989; Nag et al., 1990). Gaseous, liquid expanded (LE) and liquid condensed (LC) phases have been visualized for monolayers composed of different phospholipids (Knobler, 1990; Möhwald, 1990). Visual observation of these phases by fluorescence microscopy requires the monolayers to contain a small amount of fluorescent probe which partitions preferentially in the different phases. Visual observation of such probes have displayed the coexisting liquid condensed and liquid expanded regions, of various monolayers (Gaub et al., 1986; Knobler, 1990; Möhwald, 1990; Nag et al., 1990). Lipid-lipid (Heckl et al., 1988; Weis, 1991) and lipid-protein (Möhwald, 1990) interactions in monolayers have also been studied using this technique. Epifluorescence studies of monolayers have recently been substantiated by Brewster angle microscopy (Honig and Mobius, 1991) and x-ray diffraction (Möhwald, 1990), and supported by Fourier-transform infrared (FT-IR) observations (Dluhy, 1989).

In this study we have investigated a system of mixed monolayers of DPPC:DOPC using the epifluorescence microscopic technique. We have examined the changing sizes of condensed domains as a function of composition and surface pressure and compared our results to the thermotropic phase behavior of these lipid systems in bilayers (Phillips et al., 1970; Lentz et al., 1976). We have also sought evidence for the potential selective exclusion of one component from monolayers during a series of cycles of rapid compression to high surface pressure and re-expansion.

MATERIALS AND METHODS

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Sigma Chemical, St. Louis, MO. The fluorescent probe 1-palmitoyl-2-{12-[(7-nitro-2–1, 3-benzoxadizole-4-yl)amino]dodecanoyl}phosphatidylcholine (NBD-PC) was obtained from Avanti Polar Lipids, Birmingham, AL. The cationic probe 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO-C_{18}) was a product of Molecular Probes Inc., Eugene, OR. Stock solutions of the probes and the lipids were prepared by dissolving them in chloroform:methanol (3:1, v/v). DPPC, DOPC, and probe were mixed at molar ratios of 69:30:1, 49:50:1, and 29:70:1, and each individual lipid was mixed with probe at molar ratios 99:1 and stored at −20°C. Monolayers were spread on an unbuffered 0.15 M NaCl subphase with pH initially adjusted to 6.9. The saline was made with deionized, doubly distilled water, the second distillation being from dilute potassium permanganate.

Compression and expansion of the monolayers and surface pressure-area measurements were performed on a epifluorescence microscopic surface balance whose construction and operation are described elsewhere (Nag et al., 1990, 1991). Before spreading of the monolayer the surface was repeatedly aspirated to clean the interface of contaminants. Monolayers were spread from chloroform:methanol (3:1, v/v) solutions using a Hamilton syringe. A period of 30 min was allowed for solvent evaporation before compression and expansion were begun.

Monolayers were compressed at two different rates of 20 $mm^2\ s^{-1}$ (slow) and 600 $mm^2\ s^{-1}$ (fast) corresponding to initial rates of 0.19 and 5.6 $Å^2$ $molecule^{-1}\ s^{-1}$, respectively, for the usual surface loads. Isotherms of DPPC, DOPC, and their mixtures were obtained using both rates of compression at a temperature of 23 ± 1°C. In experiments where visual observations were performed, the monolayers were compressed in 20 steps, and the barrier stopped after each step for 1 min. During about eight of these 1-min periods at selected surface pressures, video recording of monolayer appearance was performed. Processing and analysis of the recorded video images were performed using operator interactive software (JAVA; Jandel Scientific, Cotre Madera, CA) on a personal computer as described elsewhere (Nag et al., 1990, 1991).

RESULTS

When DPPC monolayers were compressed at a fast rate (600 $mm^2\ s^{-1}$) isotherms showed onset of a LE-LC plateau at a surface pressure of ~7 mN m^{-1} and collapse at ~71 mN m^{-1}. DOPC monolayers compressed at the same rate showed no LE-LC plateau, and collapsed at 50 mN m^{-1}. When compressed at a slower rate of 20 $mm^2\ s^{-1}$ the DPPC and DOPC monolayers showed behavior essentially the same as that obtained at the fast rate. The lipids were spread at an area per molecule of 140 ± 5 $Å^2$ $molecule^{-1}$. Fig. 1 shows the isotherms obtained when monolayers of DPPC:NBD-PC 99:1, DPPC:DOPC:NBD-PC 49:50:1, and DOPC:NBD-PC 99:1 were compressed slowly with interruptions for video recording. The inset of Fig. 1 shows the isotherms of monolayers of DPPC:DOPC:NBD-PC 29:70:1 and 69:30:1 compressed at the same rate. The letters *A–E*, *a–e*, *a1–e1*, and *a2–e2* indicate the points where visual fields were recorded for analysis. Isotherms of DOPC:NBD-PC 99:1 showed only LE phase up to the point of monolayer collapse.

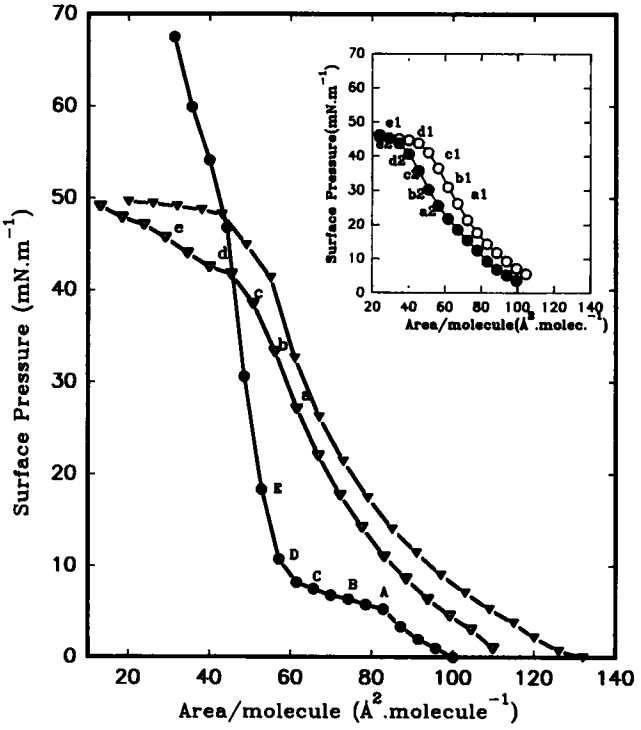

FIGURE 1 Isotherms of DPPC:NBD-PC 99:1 (●), DPPC:DOPC: NBD-PC 49:50:1 (▽), and DOPC:NBD-PC 99:1 (▼) compressed in steps at a temperature of 23 ± 1°C. Inset: isotherms of DPPC:DOPC:NBD-PC 29:70:1 (○), and 69:30:1 (●). The monolayers were compressed in 20 steps at a speed of 20 $mm^2\ s^{-1}$, and at some steps (indicated by the letters) a waiting period of one minute was introduced, during which visual recording was made.

The LE-LC phase transition of monolayers of DPPC:NBD-PC (99:1) under slow compression was observed visually (Fig. 2, *A–E*). Very small dark condensed domains were found to appear at surface pressures of 3–5 mN m^{-1}. As surface pressure was increased to 17 mN m^{-1} the domains grew to larger size. The domains were relatively homogenous in shape and distributed evenly over the visual fields. The domains came in contact with one another at surface pressures > 17 mN m^{-1}, but did not appear to coalesce or fuse up to a surface pressure of 71 mN m^{-1}. This phenomenon was also observed using the cationic fluorescent probe DiO-C_{18}. DiO-C_{18} gave more intense fluorescence in the condensed regions (dark regions with NBD-PC) than in the fluid phase (fluorescent with NBD-PC). In DOPC monolayers containing 1 mol% of NBD-PC, continuous fluorescent fields, or a LE phase, were observed from low surface pressures up to the point of DOPC monolayer collapse (~50 mN m^{-1}). At surface pressures ~2 mN/m below collapse of the DOPC monolayers, small areas (~2 μm in diameter) of intense fluorescence, were observed in addition to the general fluorescence of the whole field. It is possible that the intensely fluorescent areas could be caused by aggregation of densely packed regions of probe (probe aggregates) within the monolayer. Another potential explanation for the increased intensity could be the formation of multilayers (collapsed phase) where the amount of probe in the direction normal to the surface is increased without increasing the packing density.

Monolayers of DPPC plus DOPC containing 1 mol% NBD-PC showed different visual characteristics. For the DPPC:DOPC:NBD-PC 49:50:1 monolayers (Fig. 2, *a–e*) a uniformly fluorescent field was observed at surface pressures between 3 and 13 mN m^{-1}. For this mixture, small condensed domains appeared at a surface pressure of 14 mN m^{-1}. The domains grew in size with increasing surface pressure up to the point *d* in the isotherm. These domains were not homogenous in shape or size, and most fields observed contained domains ranging between 50 and 250 μm^2. In all mixed monolayers at surface pressures greater than 43 mN m^{-1}, three types of regions were seen. A low-intensity fluorescent background with dark condensed regions and small spots of high intensity fluorescence were typical of the images at these surface pressures. Decreasing the DPPC content of the monolayer resulted in a slight decrease in the domain size at equivalent surface pressures as shown in Fig. 2. Similar features of the monolayers were observed using the probe DiO-C_{18} except at surface pressure > 43 mN m^{-1} no areas which corresponded to the small fluorescent spots seen with NBD-PC were detected.

At surface pressures greater than 20 mN m^{-1} monolayers of DPPC:DOPC:NBD-PC 29:70:1 showed an appearance different from that of the other mixtures. The distribution of condensed domains was not uniform across the monolayer. At surface pressures > 20 mN m^{-1} large fluorescent, expanded areas, several times larger than the field of view using

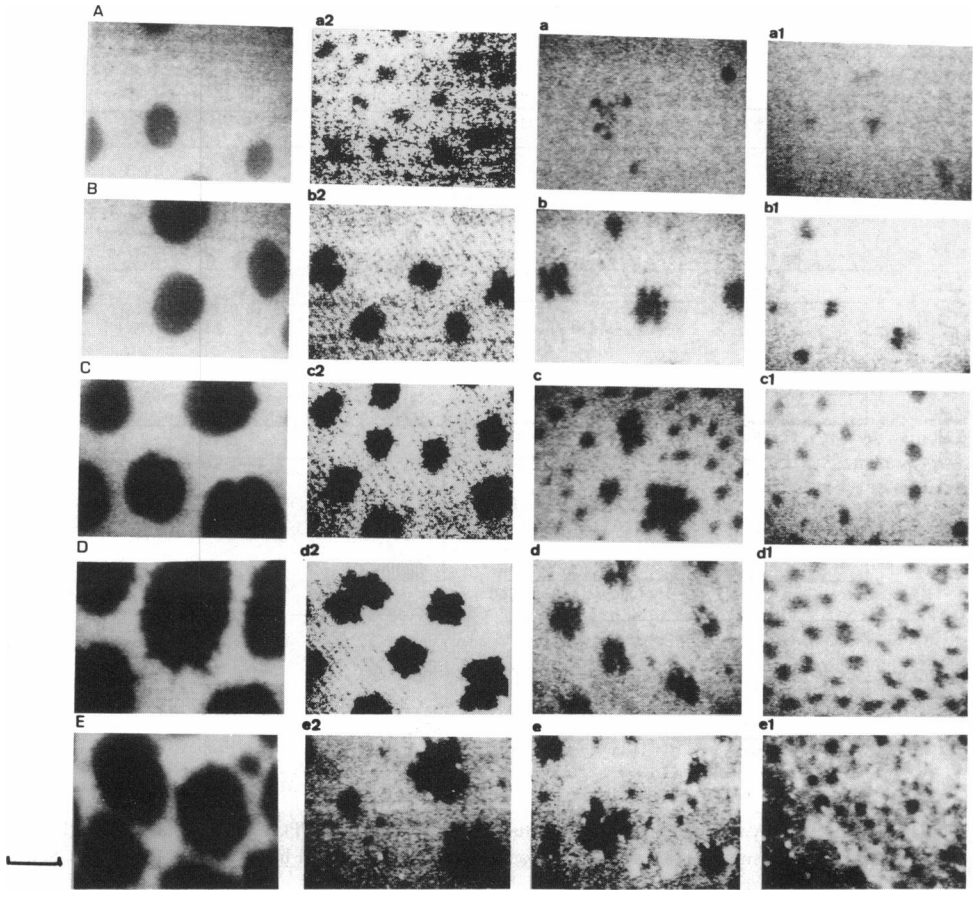

FIGURE 2 Typical images of monolayers of DPPC:NBD-PC 99:1 (*A–E*), DPPC:DOPC:NBD-PC 69:30:1 (*a2–e2*), 49:50:1 (*a–e*), and 29:70:1 (*a1–e1*) photographed from the video monitor at the surface pressures indicated in the isotherms in Fig. 1. In monolayers of DPPC:DOPC:NBD-PC 29:70:1 there were whole fields containing no condensed lipid at surface pressures between 0 and 45 mN/m. The scale bar is 25 μm.

a 40 × objective lens, coexisted together with areas where a mixture of fluorescent and dark domains were detected. This feature was confirmed under lower magnification (16 × objective). Because their distribution was so heterogenous, monolayers of this composition were subjected to limited quantitative analysis. Average sizes of the dark domains could be obtained, but their coverage of the total surface could not be ascertained. In all the mixed monolayers the visual fields observed at surface pressures above 45 mN m^{-1} were found to have a lack of contrast between the two lipid phases due to low fluorescence observed from the expanded regions.

Quantitative analysis of the appearance of the monolayer was performed on digitized recorded images. The average size and number of domains was obtained as described elsewhere (Nag et al., 1991). Six to ten randomly selected frames were analyzed at each surface pressure, indicated by letters in the isotherms in Fig. 1. Fig. 3 shows the frequency distributions of domain sizes obtained from images of monolayers of DPPC:NBD-PC 99:1, DPPC:DOPC:NBD-PC 49:50:1, and DPPC:DOPC:1 69:30:1. For DPPC:NBD-PC 99:1 (Fig. 3 *a*) the average size of the condensed domains increased with increasing surface pressure, as did the scatter of sizes of the condensed regions. This pattern was consistent with those observed previously with monolayers of this lipid (Nag et al., 1991). At surface pressures higher than 17 mN m^{-1} the domains began to come in contact with each other, and they could not be identified individually in order to perform a frequency distribution analysis. The frequency distributions for the mixed monolayers (Fig. 3, *b* and *c*) show a different pattern; between π = 20 mN m^{-1} and π = 30 mN m^{-1} the heterogeneity of size of the condensed domains increased, and above 40 mN m^{-1} they reverted to the narrower distributions observed at the low surface pressure. For mixed monolayers the range of sizes and the average size of the condensed domains was generally smaller than that seen for DPPC alone.

Fig. 4 shows the plot of average domain size as a function of surface pressure for DPPC monolayers and the three different DPPC:DOPC monolayers. For the monolayers of mixtures of lipids, the average domain size increased up to a surface pressure of 35 mN m^{-1}, and then it decreased with further increase of surface pressure. For the mixed systems, the largest condensed domains were observed in the monolayers of DPPC:DOPC:NBD-PC 69:30:1. In that mixture the domains grew to about 200 μm^2 at π = 35 mN m^{-1} and then decreased to 70 μm^2 at surface pressures of ~40 mN m^{-1}. A similar pattern in the size-pressure relationship was also observed for monolayers of DPPC:DOPC:NBD-PC 49:50:1 and 29:70:1. These patterns were reproducible with separate

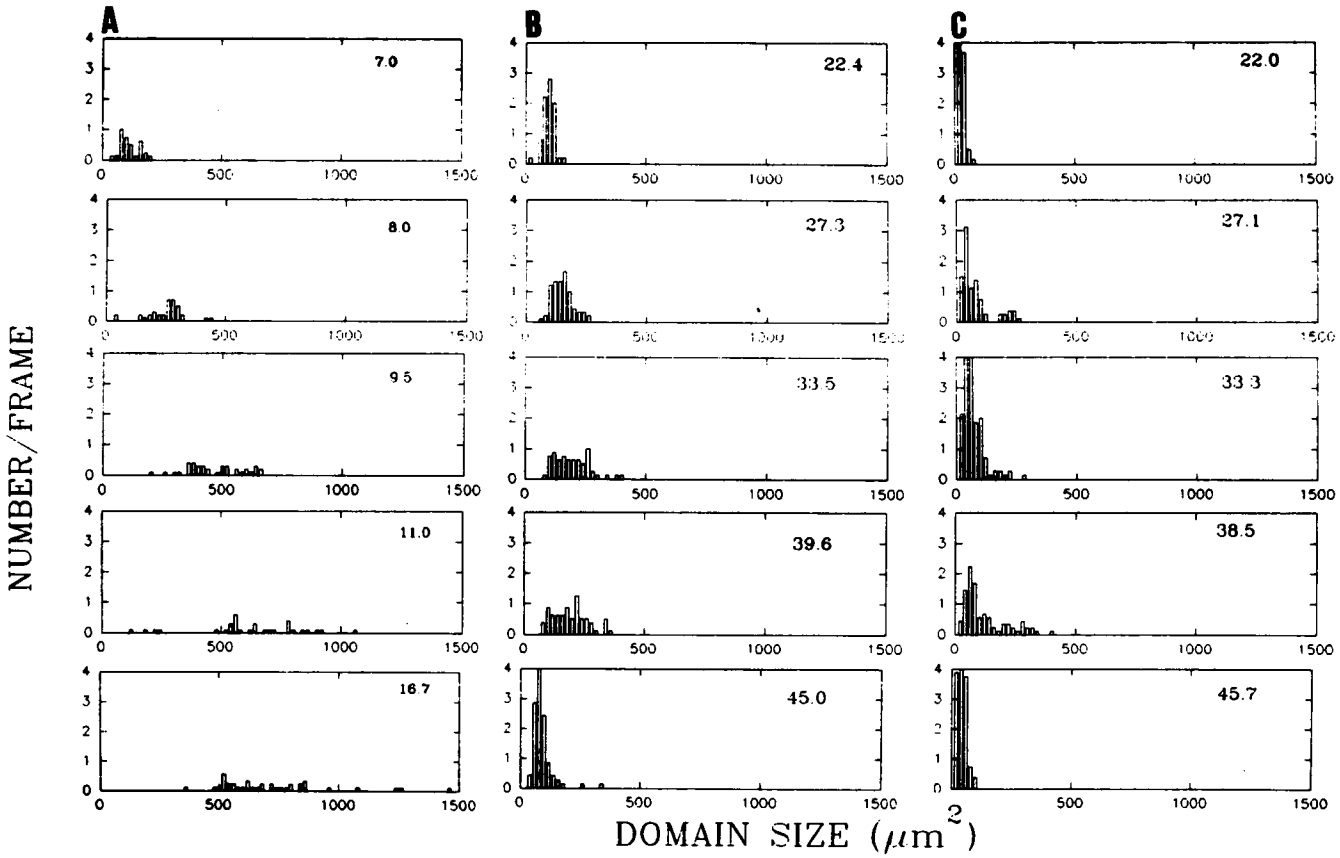

FIGURE 3 Frequency distributions of domain sizes of monolayers of DPPC:NBD-PC 99:1 (*A*), DPPC:DOPC:NBD-PC 69:30:1 (*B*), and 49:50:1 (*C*). The surface pressure in mN m^{-1} for each distribution is shown in the top right-hand corner of each panel. Six to ten randomly selected frames were analyzed for each surface pressure.

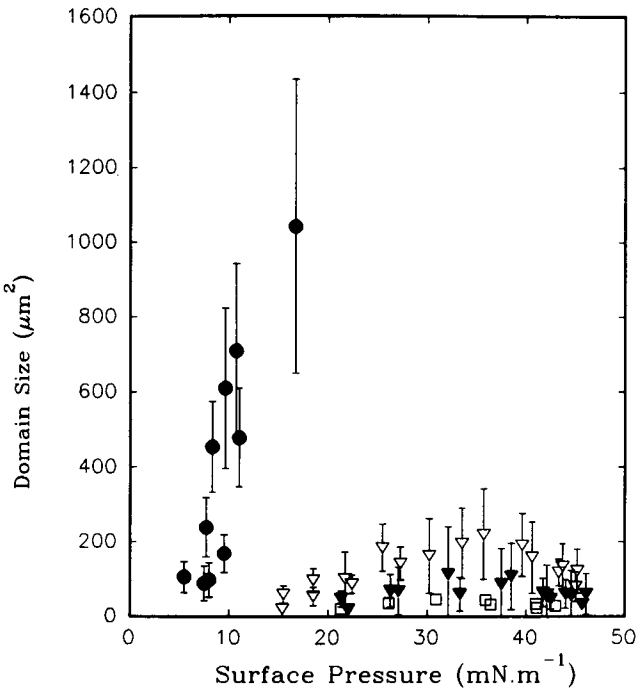

FIGURE 4 Average domain size, based on six to ten images from two separate monolayers each, of DPPC:NBD-PC 99:1 (●), DPPC:DOPC: NBD-PC 69:30:1 (▽), 49:50:1 (▼), and 29:70:1 (□) plotted as a function of surface pressure. The error bars indicate one standard deviation.

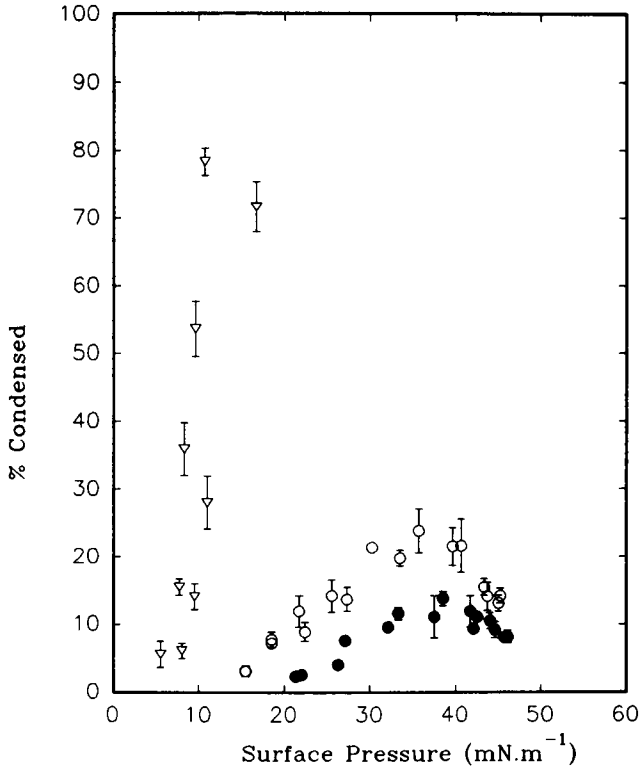

FIGURE 5 Total percentage of condensed regions per frame plotted as a function of surface pressure for DPPC:NBD-PC 99:1 (▽), DPPC:DOPC: NBD-PC 69:30:1 (○), and 49:50:1 (●) monolayers. Error bars represent one standard deviation.

mixtures of the same components. The sizes of condensed domains, including the maximum size at ~35 mN m^{-1} was smaller with increasing content of DOPC in the monolayers.

Fig. 5 shows a plot of percentage of condensed lipid ((total area of the condensed domains)/(total area of the frame) × 100) as a function of surface pressure. For DPPC:NBD-PC 99:1 monolayers, the percentage of condensed lipid increased as surface pressure was raised, reaching a value near 80% at π = 17 mN m^{-1}. A similar proportion of condensed lipid was observed in DPPC monolayers at a similar temperature that were compressed at a slower rate and which contained a slightly lower nominal probe concentration (0.7 mol%) by Flörsheimer and Möhwald (1989). If one assumes that the probe is primarily soluble in the liquid expanded phase, its concentration in that phase would then be approaching 5 mol%, and it would have an effect to preserve a greater amount of the lipid in the fluid state than would be the case in the absence of probe. It is of interest, however, that the proportion of condensed lipids in monolayers containing DPPC:DOPC:NBD-PC 69:30:1 and 49:50:1 showed a similar pattern to the average sizes. Over the range of 15 to 35 mN m^{-1} the percent condensation rose with increasing surface pressure. The percent condensation for monolayers containing DPPC:DOPC:NBD-PC 29:70:1 could not be calculated because of heterogenous distribution of frames noted above. One can only estimate that the total percentage of condensed domains in the monolayer was low.

Visual observations were performed on DPPC:DOPC: NBD-PC 49:50:1 monolayers under repetitive dynamic conditions of compression-expansion recycling. The monolayers were spread at an initial surface load corresponding to ~80 Å^2 molecule^{-1}, and they were repeatedly compressed and expanded 10 times at a fast rate of 600 mm^2/s (or an initial rate of 3.2 Å^2 molecule^{-1} s^{-1}). An 11th compression was done slowly in steps (20 mm^2/s) and visual observation was performed for 1 min at each step. The isotherms of the fast compression-expansion cycles are shown in the inset of Fig. 6 *a*. Analysis of the images observed on the 11th compression compared to the data obtained from a initial slow compression of DPPC:DOPC:NBD-PC 49:50:1 monolayers showed an increase in the percentage of condensed lipids on the eleventh compression at equivalent surface pressures (Fig. 6 *a*). The maximum percentage of condensed lipid (obtained near π = 35 mN m^{-1}) increased nearly threefold (from about 12% to about 35%) between the singly and the 11-times-compressed monolayer. Fig. 6 *b* shows typical images observed at π ~33 mN m^{-1} from a singly and an 11-times-compressed monolayer of DPPC:DOPC:NBD-PC 49:50:1. The number of condensed domains per frame was increased substantially on the 11th compression, but sizes of the condensed domains were not substantially changed between the first and 11th compressions.

DISCUSSION

Fluorescence microscopy has been used in studying monolayers of various lipids and cholesterol-phospholipid and

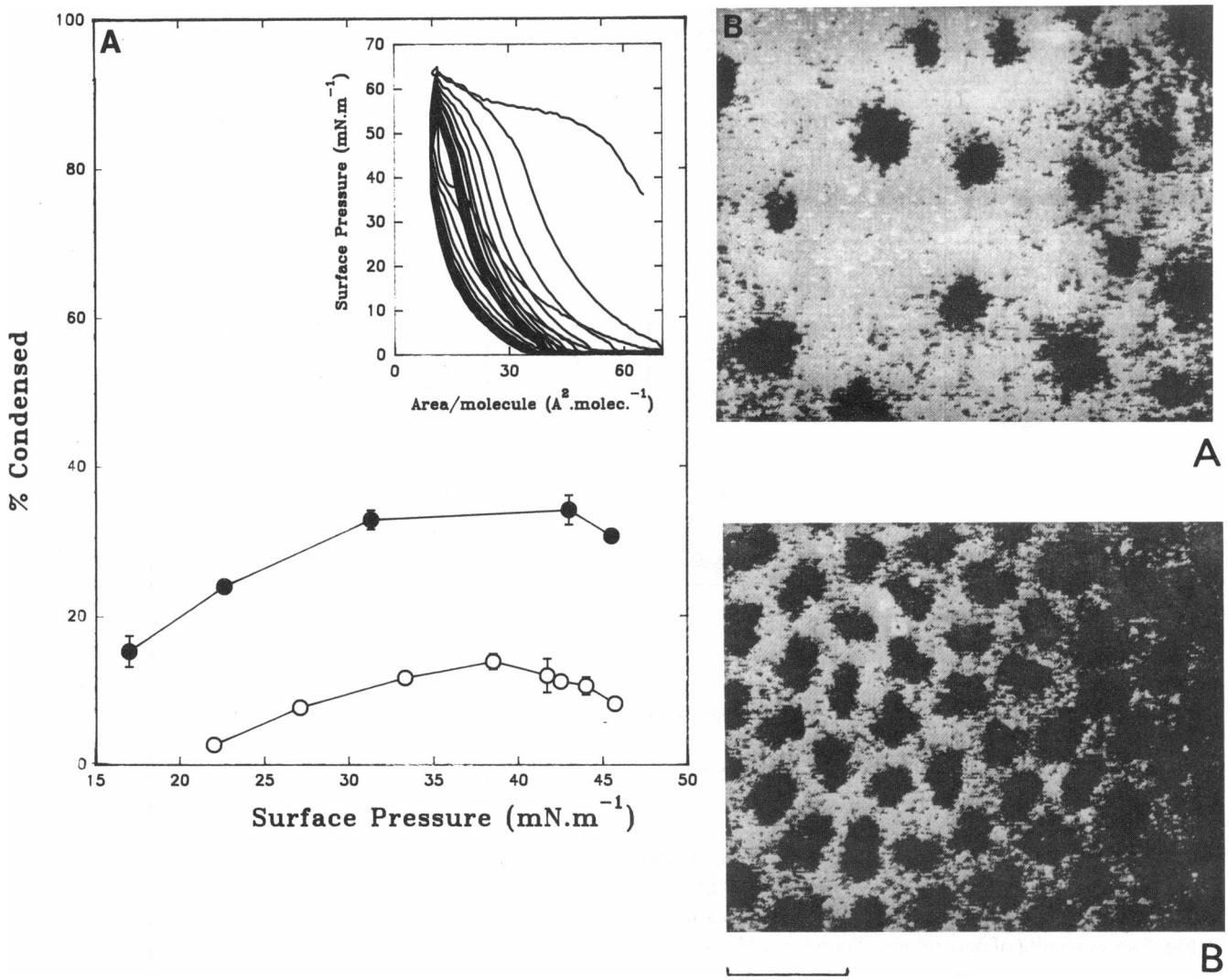

FIGURE 6 (*A*) Percentage of condensed regions as a function of surface pressure for two DPPC:DOPC:NBD-PC 49:50:1 monolayers, one compressed slowly once at 20 mm^2 s^{-1} (○), and the other compressed 11 times (fast (●) at 600 mm^2 s^{-1} for 10 cycles, and followed by a slow compression on the 11th cycle). The isotherms of the 11 compression-expansion cycles are shown in the inset. (*B*) Typical images at a surface pressure ~33 mN m^{-1} from a single slow first compression (*A*) and the 11th (slow) compression (*B*) of a monolayer of DPPC:DOPC:NBD-PC 49:50:1. Scale bar is 25 μm.

lipid-protein mixtures (Möhwald, 1990). In DPPC monolayers studied here there was a well defined LE-LC phase transition and formation of large condensed domains with increasing surface pressure, a behavior consistent with that seen in previous studies (Nag et al., 1991). DOPC showed a fluid or LE phase with no LE-LC phase transition up to the point of monolayer collapse. This behavior of DOPC in monolayers has also been reported by others using a different lipid probe in the monolayer (Yu and Hui, 1991). Recently electron microscopy of DOPC monolayers transferred to solid substrate showed a single homogenous phase up to the point of monolayer collapse (Tchoreloff et al., 1991).

The surface pressure-area isotherms for the monolayers of mixtures of DPPC and DOPC did not show any inflections or plateau regions at low to intermediate surface pressures where LE-LC transitions occur in pure DPPC monolayers, although collapse plateaux were observed at higher pressures. Condensed domains did form in the mixed monolayers, but the domain sizes and total amount of condensed lipid were smaller than those observed in monolayers of DPPC alone. In the mixed monolayers the condensed domains did not appear until higher surface pressures were attained than those required to produce condensed domains in monolayers of DPPC. Even in monolayers containing 69 mol% DPPC the domains grew to a average maximum size of ~200 μm^2 at π ~35 mN/m^{-1} compared to ~1100 μm^2 in monolayers of DPPC plus probe at substantially lower surface pressures (π near 17 mN m^{-1}). Also, the percent of total condensed phase of the mixed monolayers never reached more than an average of about 25%. In the mixed monolayers it is presumed that the condensed domains are enriched in DPPC over the original proportions of that lipid. This behavior is consistent with the broad thermal transitions of DPPC-DOPC mixtures in bilayers which show monotectic properties (Phillips et al., 1970; Egberts et al., 1989).

The monolayer properties can be related to the phase properties of bilayers of these lipids (Lentz et al., 1976). At 21°C DOPC is well above its gel to liquid crystal phase transition

temperature, whereas DPPC is well below it. Thus, monolayers of DOPC never showed condensed domains at any pressure, whereas DPPC films undergo the LE to LC transition, showing condensed domains, at relatively low pressure. The mixtures approximating DPPC:DOPC 3:7, 5:5, and 7:3 are all in the coexistence region of gel plus liquid crystal for bilayers at 21°C. The first of these three mixtures is very close to the liquidus of the bilayer phase diagram at 21°C, and the appearance of only very small condensed domains in the corresponding monolayer is consistent with the mixture not readily forming condensed domains. The other two mixtures show condensed domains consistent with their locations in the bilayer phase mixtures. The similarity of the properties of the monolayers and bilayers would suggest that it is reasonable to assume that the condensed domains in the monolayers are enriched in DPPC.

Recently electron micrographs of DPPC:DOPC 50:50 monolayers near collapse pressures have been observed to show patchy areas 10-Å thicker than those of the background monolayer (Tchoreloff et al., 1991). The patches have been suggested to be representative of rigid hydrocarbon regions coexisting with fluid areas (Tchoreloff et al., 1991).

The domain sizes in monolayers are influenced by the rate of monolayer compression with faster rates tending to give smaller domain sizes (e.g., Nag et al., 1991; Shimomura et al., 1992). The slow rate used here was the same as that used by us before to give properties for DPPC that resembled those seen by others using similar or somewhat slower rates (Nag et al., 1991). We therefore assume that the smaller domains seen in the mixed films are a consequence of their equilibrium properties, that is, the domain sizes are those expected from the phase diagram (see above), and that they are not small because of diffusion-limited domain growth. Two of the mixtures containing 30 and 50% DOPC were examined in separate experiments and they gave domain size distributions consistent with those seen in Fig. 3. While the properties of the fluid and rigid domains in the mixed monolayers were consistent with what might be expected from the bilayer phase diagram, the exact sizes of the condensed domains could also be to some extend influenced by the probe concentration. A future study of the influence of probe concentrations on the sizes of the domains would perhaps be useful. Currently technical restrictions on sensitivity prevent us from carrying out a detailed investigation of this phenomenon at much lower probe concentrations.

In all the mixed monolayers studied the average size of the condensed domains increased up to a surface pressure of 35 mN m^{-1} and then decreased with higher pressure. The domain sizes and the percentages of condensed domains were also affected by the amount of unsaturated lipid in the monolayers. As would be expected, for any given π, larger condensed domains and greater total coverage with condensed domains were correlated with increasing DPPC concentration in the mixed monolayers.

At surface pressures higher than 35 mN m^{-1} the decrease of domain size and the total condensed lipids indicates that a more complex process of lipid mixing, and particularly probe mixing, and chain reorientation may be occurring. A process of liquid ordering of DOPC could be consistent with the observation that, above π = 35 mN m^{-1}, the mixed monolayers showed fluorescent portions with reduced intensity together with very small (2 μm^2) regions of intense fluorescence. Above pressures of 35 mM m^{-1} also, the sizes of the condensed domains decreased in the mixture with 69% DPPC. The percentage of the total fields covered by condensed domains also decreased for the mixture containing 69 and 49% DPPC. As the condensed domains initially nucleate under pressure, they would be rich in DPPC. As further condensation occurs, however, sufficient DOPC may be occurring in the condensed regions to cause probe to be dissolved in them. This would mean that visual differentiation between outer portions of condensed regions and the more fluid regions would become difficult. Such regionalized condensed domains might not be at equilibrium, since kinetic barriers to diffusion of the lipids and probes may be high enough to prevent equilibration over the times of observation used here (Nag et al., 1991). A second explanation is that partitioning of probe between the condensed and expanded phase of the mixed monolayer is pressure dependent, and that above 35 mN m^{-1} a change in partitioning of the probe plus kinetic limitations to its diffusions causes the apparent decrease in dark, condensed phase. A third explanation is that at higher compressions the fundamental mixing properties of the probe-containing and probe-depleted phases are altered so that a new system is being described in this high pressure region.

At the usual temperature of biological organisms their membranes consist mainly of fluid and, occasionally, some rigid lipids. Such systems are complex in their phase properties. This study suggests that in mixed monolayers of DPPC:DOPC at pressures feasible in membranes condensed regions coexist with a partially ordered fluid phase. Such partial ordering of charged fluid lipids into small areas in single leaflets of bilayers has been observed in small unilamellar vesicles by fluorescence microscopy (Haverstick and Glasser, 1989). In that case the regionally ordered areas were induced by cytochrome *c* or calcium ions (Haverstick and Glasser, 1989).

Pulmonary surfactant is a mixture of saturated and unsaturated lipid plus proteins. These results indicate that the amount of condensed phase which can occur if there is unsaturated lipid present is much less than one expects on the basis of loss of components. In simple models of pulmonary surfactant where unsaturated and saturated components are mixed (e.g., Hawco et al. (1981b) and this work), the small amounts of condensed regions may explain why, under slow compression, the monolayers collapse before high π is reached.

Recycling of equimolar mixed monolayers for a number of cycles at a fast speed resulted in an appearance that was consistent with a loss of unsaturated lipids from the monolayer. The percent of condensed lipids in the fields after 11 cycles of fast barrier movement was increased about threefold over that of a monolayer compressed only once. The change in the properties of the film might result from the repeated cycling causing an annealing of the film such that

film defects which prevent the formation of condensed phase are reduced, leading to a more highly condensed and more stable film. A second interpretation suggests that during cyclic compression and expansion some lipids which dissolved the probe were being excluded from the monolayer. The exclusion may involve both lipid species, but the unsaturated lipids would appear to be squeezed out to a greater extent than the saturated lipid, since the threefold increase in the percentage of condensed domains is likely a good marker of the increased saturated lipid content in the monolayers after cyclic compression. Preferential squeeze-out of the unsaturated lipid species has been suggested to occur in similar mixed monolayers under rapid compression (Notter et al., 1980; Hawco et al., 1981a; Egberts et al., 1989) and it may be an important factor in function of pulmonary surfactant. This finding is consistent with the presumption that pulmonary surfactant is refined in the surface by selective exclusion of unsaturated species (Hildebran et al., 1979; Notter et al., 1980; Hawco et al., 1981a). It is most interesting to note that Schürch et al. (1989), using whole natural surfactant in a leak-free, captive bubble balance, find that one slow compression can yield bubble shapes consistent with the presence of a monolayer highly enriched in DPPC. The composition of the surfactant may lead to a more effective selective exclusion of non-DPPC components than seen in this simple model. Such a process in natural surfactant could be promoted not only by its special lipid composition, but also by the presence of unique proteins (Curstedt et al., 1987). It is also possible, however, that the unique properties of the monolayer formed in the presence of natural surfactant reflect selective insertion of materials into the monolayer rather than selective exclusion after formation. The results presented here suggest that the latter explanation should not be eliminated from the possible explanation of properties of pulmonary surfactant as yet.

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