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Nonselective squeeze-out of dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol from binary mixed monolayers with dipalmitoylphosphatidylcholine

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In order to enable the possible use of dipalmitoylphosphatidylcholine as an artificial lung surfactant, the addition of dioleoylphosphatidylcholine or dioleoylphosphatidylglycerol has been suggested. A preferential loss of molecules of the second component during compression of the interfacial layer was proposed. In this study two types of measurement were carried out in order to verify such a preferential squeeze-out. In the first type, electron micrographs of a pure dipalmitoylphosphatidylcholine monolayer and of mixed monolayers of dipalmitoylphosphatidylcholine and egg phosphatidylglycerol were taken in order to study the nature of the structures formed during compression of the monolayer. The electron microscopy photos show horizontally stacked layers in the collapse phase of dipalmitoylphosphatidylcholine, and long vertical ridges in the mixed monolayers up to 20% second component. At higher concentrations of the second component no such structures can be detected. The second type involved monolayer studies with binary mixtures of dipalmitoylphosphatidylcholine and dioleoylphosphatidylcholine or dioleoylphosphatidylglycerol, one of the pair being always radioactively labelled. Counting the radioactivities in bulk phase and monolayer after compression revealed nonselective squeeze-out of either component.

Introduction

Pulmonary surfactant is a phospholipid-protein complex present in the alveoli of the mammalian lung [1]. Dipalmitoylphosphatidylcholine (DPPC) is highest in concentration [2] and is the very component which allows a monolayer of pulmonary surfactant to reduce surface tension to very low values [3]. However, DPPC cannot spread out spontaneously to form a monolayer at physiological temperatures (37–38°C), whereas lung surfactant does so readily [3,4]. To explain this different behavior, the hypothesis of preferential squeeze-out has been introduced [5,6]. According to this concept the unsaturated lipids serve as agents which lower the transition temperature from gel-to-liquid-crystal, making spontaneous spreading possible. However, the unsaturated lipids will also lower the collapse surface pressure to below the required value. The concept of preferential

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

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squeeze-out tries to offer a solution for this dilemma by stating that the unsaturated components of lung surfactant are preferentially squeezed out from the monolayer during compression [5,6]. This process should enrich the monolayer with respect to DPPC and raise its surface stability [7].

Boonman et al. [8] reported the observation of squeeze-out from mixtures of DPPC with either egg PC, soy PE and soy PI, appearing as a plateau in the plot of surface tension vs. surface area (σ–A; see Fig. 1). Similar plateaux are observed by De Fontagnes et al. [10]. Boonman et al. [8,9] report that squeeze-out of DPPC has to be assumed to account for the number of molecules squeezed out, as estimated from the length of the plateau. This does not support the concept of preferential squeeze-out.

In this paper we present results of additional experiments. In preliminary experiments we took micrographs of monolayers of DPPC and DPPC-egg PG mixtures in order to investigate the nature of squeeze-out during plateau formation. In addition, we assessed the amount of molecules expelled to the subphase during compression of binary-mixed monolayers constituted by ^{14}C-DPPC and DOPC, DPPC and ^{14}C-DOPC, and ^{14}C-DPPC and DOPG, respectively. The experiments with the ^{14}C-DOPC were chosen because ^{14}C-DOPG was not commercially available.

Materials

DPPC, DOPC and DOPG were purchased from Sigma Chemical Company (St. Louis) while ^{14}C-DPPC and ^{14}C-DOPC were from New England Nuclear (Dreieich). Solutions of ^{14}C-DPPC and ^{14}C-DOPC were made with an activity of 0.1–1 μCi/mol by adding non-radioactive DPPC or DOPC. The concentration was 1 μmol/ml. 1 μmol/ml solutions of (unlabelled) DPPC, DOPC and DOPG were also made. From these solutions mixtures were made of DPPC/DOPC and DPPC/DOPG with different molar ratios. In all mixtures only one component was labelled.

Methods

Surface balance

Surface tension as a function of monolayer area

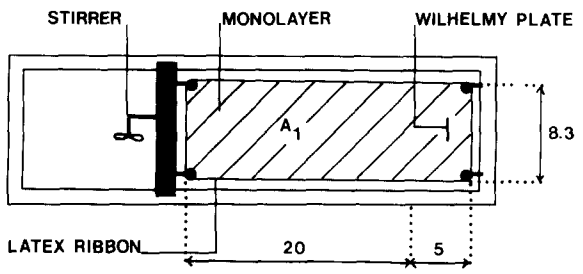

Fig. 1. Modified Langmuir-Wilhelmy surface balance with elastic latex ribbon. A_1, initial surface area; dimensions are given in cm.

was measured with a modified Langmuir-Wilhelmy surface balance developed at our laboratory. The subphase was contained in a teflon trough; an elastic band, consisting of natural latex and cleaned in a Soxhlet apparatus filled with ethanol and partially submerged in the subphase, enclosed the monolayer surface area. It was checked that after the cleaning procedure no more impurities were released from the band. The construction made leakage of monolayer molecules virtually impossible (Gieles, P.M.C., unpublished results). The monolayer is compressed and expanded by means of a moving barrier which stretches and relaxes the elastic band (Fig. 1).

The surface tension was measured with a platinum Wilhelmy plate electrolytically platinised in order to improve wetting, and suspended from an electrobalance (Cahn RH).

The trough and Wilhelmy balance were located in a temperature-controlled climate box saturated with water vapour; the temperature was monitored (AD 590, Philips) below and as close as possible to the air/water interface and kept at 37.0 ± 0.2°C.

The aqueous subphase was prepared from triple-distilled water containing 0.01 M Tris-HCl (pH 7.0).

Monolayers were spread from chloroform solutions of the phospholipids with a digital Transferpettor micropipette (0–50 μl, 0.1 μl adjustable). The spread quantity was 50 nmol for all measurements.

Compressions were carried out from 207.5 cm^2 (100%) to 20% of the initial surface area with a rate of 0.51 cm^2/s. Isothermal plots of σ versus A were obtained on an X-Y recorder. During the

experiments the subphase was stirred in order to distribute the molecules which are squeezed out into the subphase (Fig. 1).

Recovery procedures

After compression to 20% of its initial surface area, the monolayer was aspirated into a counting vial (M). Some of the subphase (1–2.5 ml) was inevitably aspirated with the monolayer. The entire subphase (340 ml) was recovered by aspirating the subphase outside the monolayer area into a flask. This procedure was followed in order to avoid inhomogeneity in sampling the subphase. By adding scintillation liquid (New England Nuclear 263), which acts as a detergent, the solution was made homogeneous. From this flask we took a sample of 2 ml and put it into a second counting vial (S).

Third, a reference sample (R) was made, containing the same amount of phospholipid mixture as was used for spreading. The activity of the three samples, each containing 11 ml of scintillation liquid, was assessed in a liquid-scintillation detector (Packard 3000). The efficiency of counting, that is the ratio of counts to desintegrations per minute, is influenced by quenching. Differences in efficiency of the countings were corrected by the use of the quenching calibration curve.

Definition of molecular loss

When a monolayer is compressed to its closest packing density, molecules disappear into the aqueous subphase (squeeze-out) or form three-dimensional aggregates at the interface (collapse) and are no longer part of the monolayer. All these molecules are assumed to be 'lost' (apart from a possible respreading of molecules during expansion).

Fig. 2 shows an example of a compression curve of a binary monolayer. The surface tension remains constant during part of the compression and we assume that the surface-area variation (ΔA) for the duration of the plateau (where collapse or squeeze-out occurs) is a measure of the molecular loss (ML). For molar concentrations of DOPC and DOPG $\leqslant 20\%$ we found a second plateau at the level at which DPPC normally collapses.

If we assume that the mean area per molecule is

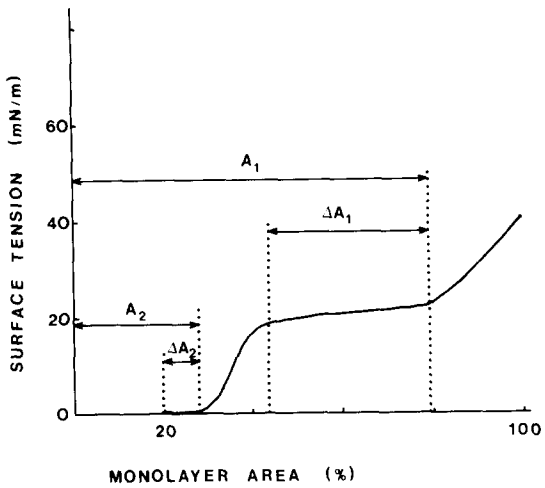

Fig. 2. Example of a $\sigma - A$ plot taken from a DPPC/DOPC (85 : 15) monolayer. Squeeze-out occurs during the first plateau (onset area A_1, 'length' ΔA_1); the second plateau, if present, implies collapse (onset area A_2, 'length' ΔA_2).

constant for the duration of the plateau it can be seen that the relative molecular loss can be given as:

$$\mathrm{ML} = \frac{\Delta A_1}{A_1} + \left(1 - \frac{\Delta A_1}{A_1}\right)\frac{\Delta A_2}{A_2} \qquad (1)$$

From the σ–A plots we cannot conclude whether the molecules stay at the surface or disappear into the aqueous subphase during compression. However, from the counting data of sample S we can calculate the number of molecules lost to the subphase.

Electron microscopy

The technique used for the electron-microscopy study of monomolecular films was similar to that described by Ries [11]. A collodion-covered grid was mounted 2 mm beneath the interface. The surface level was rapidly reduced after compression of the monolayer to collapse pressure. The monolayer was deposited on the grid and subsequently shadowed with Pt/C.

Results

The results of measurements on binary mixtures of DPPC/^{14}C-DOPC, ^{14}C-DPPC/DOPC

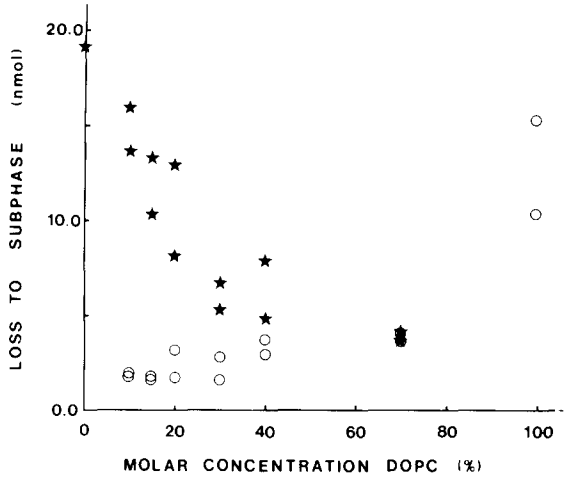

Fig. 3. Radioactivity present in the subphase after squeeze-out from DPPC/DOPC monolayers as a function of initial molar concentration DOPC. ○, DOPC loss from DPPC/^{14}C-DOPC monolayers; ★, DPPC loss from ^{14}C-DPPC/DOPC monolayers. Each point represents one compression experiment with 50 nmol the total amount spread.

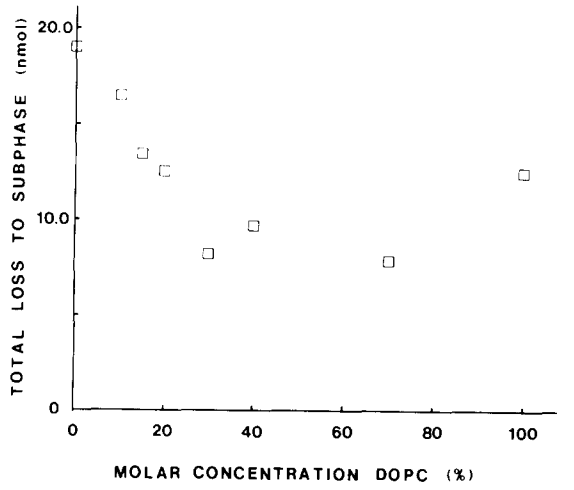

Fig. 4. Total loss of DPPC + DOPC to subphase versus initial molar concentration DOPC. This plot is the sum of the mean values for the DPPC and DOPC losses as presented in Fig. 3.

and ^{14}C-DPPC/DOPG monolayers are plotted in Figs. 3–6. All measurements were performed in duplicate at molar concentrations of DOPC or DOPG of 10, 15, 20, 30, 40, 70 and 100%.

Fig. 3 shows the results of DPPC/^{14}C-DOPC monolayers, measuring the amount of DOPC (in nmol) found in the subphase as a function of the initial molar concentration DOPG. The loss of DOPC from mixed monolayers of DPPC-DOPC containing 20 to 70 mol% DOPC increases from 2.4 to 4.0 nmol. This is 24 and 11.4%, respectively, of the DOPC initially present at the interface. Similar results were obtained for the loss of DPPC from mixtures of ^{14}C-DPPC/DOPC, (Fig. 3). The loss of DPPC from mixed monolayers of DPPC-DOPC containing 20 to 70 mol% DOPC decreases from 10.4 to 3.8 nmol. This is 26 and 25.3%, respectively, of the DPPC initially present at the interface.

Fig. 4 shows the total amount of molecules (DPPC + DOPC) lost to the subphase, the sum of both contributions in Fig. 3. The sum of DPPC + DOPC loss from mixtures containing 20 to 70% DOPC is between 12.4 and 7.9 nmol, which equals 24.8 and 15.8%, respectively, of the total amount originally spread at the interface.

Fig. 5 gives the results of mixtures of ^{14}C-DPPC/DOPG. The loss of DPPC from a mixture of DPPC/DOPG containing 20 to 70 mol% DOPG is 12.4 and 5.9 nmol, respectively. The loss of DOPG could not be measured, as the labelled compound was not available.

In Fig. 6 the molecular loss calculated from σ–A plots using formula 1 is shown. (Since this loss was similar for both DPPC/DOPC and DPPC/DOPG mixtures – at the same concentra-

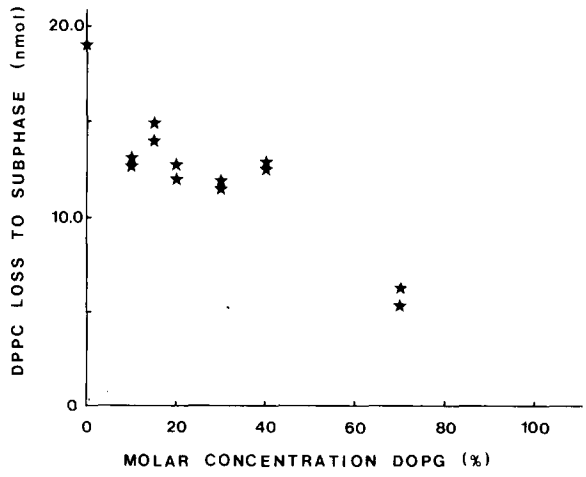

Fig. 5. The amount of radioactivity found in the subphase after squeeze-out from ^{14}C-DPPC/DOPG monolayers as a function of initial molar concentration DOPG.

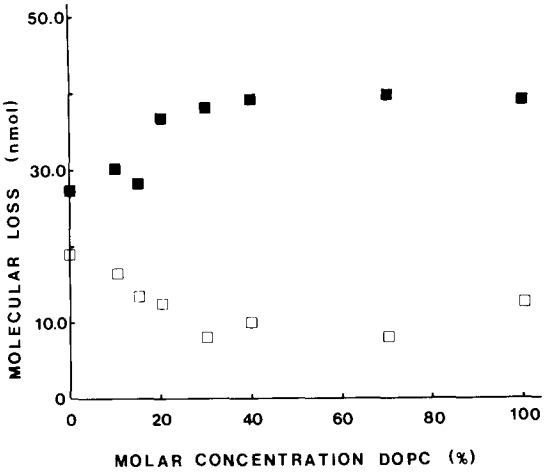

Fig. 6. Molecular loss as calculated from the $\sigma - A$ curves according to Eqn. 1 (■) in comparison with the total molecular loss as found by counting radioactivity in the subphase (□) (Fig. 4). Both are given as a function of initial molar concentration DOPC. The difference between both cases indicates the amount of monolayer material that is squeezed out but remains at the surface. Experiments with DOPG give the same results.

tion – only one plot is given). This calculated loss is compared with the loss found in the subphase (see also Fig. 4).

Squeeze-out and collapse losses, inefficiency of the monolayer recovery procedure, and desorption from the elastic band of previously adsorbed material may all contribute to the counted subphase radioactivity. We therefore carried out separate measurements with DPPC and DOPC in which the contributions of possible sources of error were examined.

The measurements showed that no lipids entered the subphase during spreading of the monolayer. It was also found that molecules were adsorbed to the elastic band. The average uptake was 6.5% for DPPC and 2% for DOPC. The adsorbed material is not believed to respread from the band when the monolayer is being aspirated, since pores in the band become smaller during compression.

Aspirating the monolayer at A_{min} was found to have a limited efficiency. Two samples were taken from the subphase: the first before, the second after aspiration of the monolayer and homogenizing of the subphase. The second sample, contain-

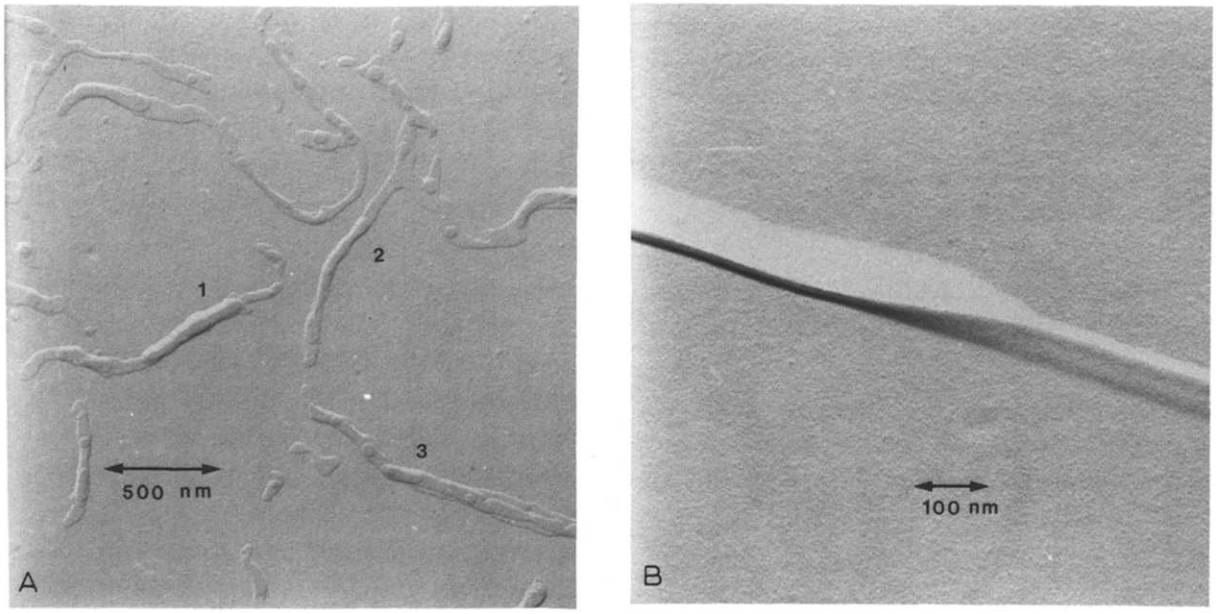

Fig. 7. (A) Electron microscopy picture of a pure DPPC monolayer compressed into collapse. The figures 1, 2, 3 refer to structures with different numbers of stacked layers. (B) EM picture of a DPPC/egg PG (80:20) monolayer compressed into its first plateau at 49 mN/m.

ing nonaspirated monolayer material, showed a significantly higher radioactivity. This procedure indicated an aspiration efficiency of about 55% for DPPC. In the case of DOPC we found an average efficiency of 88%. The total recovery (monolayer, subphase and elastic band) for these control measurements was 0.91 ± 0.04 ($n = 7$).

EM pictures

Compression of a pure DPPC monolayer to pressures of 70 mN/m results in formation of collapse structures as illustrated in Fig. 7A: long ridges compsed of several stacked layers. No such collapse structures could be detected in a pure DPPC monolayer when compressed to 50 mN/m.

Compression of mixed monolayers of DPPC-20 mol% egg PC to pressures of 49 mN/m leads to the formation of upstanding double layers (Fig. 7B right-hand side) which can fall over (Fig. 7B left-hand side). No such collapse structures could be detected in mixed monolayers of DPPC-30 mol% egg PC.

Discussion

In general the compression curve of a binary mixed monolayer may reveal two plateaux [8,10]. These plateaux (squeeze-out and collapse, respectively) are believed to reflect the loss of molecules from the monolayer. This raises the following questions: which molecules are squeezed out, where are they located and what is the structure of the mixed monolayer? We shall try to answer these questions with the help of our results.

Which molecules are squeezed out?

When the concentration of second component (DOPC or DOPG) is 20 mol% or less, two plateaux are present: the first one at the level of the collapse pressure of the less stable component, the second one at the level DPPC normally collapses. From Fig. 3 it can be seen that at these concentrations both DPPC and DOPC are found in the subphase. The presence of DPPC could be attributed to the occurrence of its own collapse plateau. However, in the experiments where the initial concentration of second component was raised to 30 mol% or higher, the second plateau is no longer detectable while DPPC was still found in the subphase (Figs. 3 and 5). So it has to be concluded that DPPC can also be squeezed out in the first plateau, and apparently this loss is caused by the presence of the second component. The loss of DPPC during the first plateau is also confirmed by the total loss calculated from the compression curves. At concentrations of second component between 30 and 70 mol% this is approx. 40 nmol (Fig. 6) which is always more than the amount of second component initially present.

The squeeze-out of DPPC does not necessarily imply that the unstable second component is only partially squeezed out. From the data presented in Figs. 3 and 6 it can be calculated that at DOPC concentrations of 20 mol%, the second plateau can only be reached assuming that all DOPC is squeezed out. Part of the DOPC molecules squeezed out of the monolayers apparently remain at the interface.

At concentrations of second component ranging from 30 to 70 mol% the total loss calculated from the σ–A curves reads 40 nmol. Since this is considerably more than the content of unstable component, a squeeze-out of all DOPC molecules may well be possible.

Where are the lost molecules located?

Apparently, both DOPC and DPPC are squeezed out and are present both in subphase and interface. When the content of second component is raised beyond 30 mol%, the first plateau is the compression curve is increased in length. A comparison between the molecular loss calculated from the compression curves and the loss calculated from the radioactivity in the subphase (Fig. 6) learns that an increasing amount of molecules remains at the interface. The reason for this change in location of the squeeze-out products is still unclear. It should be noted that during the squeeze-out process the monolayer composition may drastically change. In turn, the actual process may also be determined by the initial concentrations.

The squeeze-out products at the interface may be of physiological relevance, since respreading is believed to occur more rapidly from structures at the interface rather than from the subphase.

DOPG gives similar results as DOPC, with the dinstinction that slightly more DPPC is found in

the subphase after compression of DPPC/DOPG monolayers.

The structure of the monolayers

The EM pictures show that horizontally stacked layers can be seen at the interface at the collapse pressure of pure DPPC (70 mN/m) and that mixed monolayers of DPPC-20 mol% egg PG initially form vertically stacked layers when compressed to 49 mN/m. At this concentration of PG the second collapse pressure of 70 mN/m can still be reached (see Fig. 2). It can be envisaged that the latter structures will respread after expansion of the monolayer. Furthermore, the stacked layers at an interface may explain the difference between the measured and the calculated loss in Fig. 6. At a concentration of 30 mol% egg PC, no interfacial structures could be detected at the collapse pressure of 49 mN/m. We assume that other structures are formed at this concentration. It seems that the disappearance of the second plateau in the σ–A curve is accompagnied by (or the reflection of) a structural change in the monolayer. This may be the reason for the increase in squeeze-out products remaining at the interface.

The structures as seen on the EM pictures were not believed to be due to artefacts. The reason for that is that monolayers of different composition yielded different EM photos, while repetitive experiments yielded the same EM photos.

Experimental procedure

In spreading mixtures of DPPC-DOPC or DPPC-DOPG no loss is found due to spreading. Small amounts of adsorbed material on the latex ribbon were observed, but there was no evidence of desorption from the band or loss during parts of the compression period other than the plateau(x). The control measurements showed that squeeze-out and/or collapse, and the monolayer recovery inefficiency were the main sources for the subphase radioactivity counted.

In case of a pure DPPC monolayer this inefficiently caused largely the loss to the subphase. This poor efficiency is believed to be caused by the solid collapse structures of DPPC (see Fig. 7A) that hardly respread [3]. In mixed monolayers the solid structure is weakened by the presence of a second component. For such more fluid monolayers a much higher recovery is found. Since our conclusions are based on mixed monolayers, they are not essentially affected by the limited recovery efficiency of DPPC. Moreover, the conclusion of DPPC being lost during the first plateau is also supported by the total loss calculated from the compression curves, as was stated before.

Conclusions

From σ–A curves of, and radioactivity measurements on binary mixtures of DPPC with DOPC or DOPG it is concluded that DPPC may also be squeezed out of the monolayer in cases where the collapse pressure of DPPC is not reached. EM pictures show a significant change in the structure of the monolayer with increasing amount of second component. Most of the squeeze-out structures seem to remain at the interface.

The results indicate that under the conditions here described there is no evidence for preferential squeeze-out of the least-stable component.

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