# Distinct Steps in the Adsorption of Pulmonary Surfactant to an Air-Liquid Interface

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ABSTRACT To investigate the mechanisms by which vesicles of pulmonary surfactant adsorb to an air-liquid interface, we measured the effect of different phospholipids and of their concentration both in the subphase and at the interface on this process. Adsorbing vesicles contained the hydrophobic surfactant proteins mixed with the following four sets of surfactant phospholipids that varied the content of anionic headgroups and mixed acyl chains independently: the complete set of purified phospholipids (PPL) from calf surfactant; modified PPL (mPPL) from which the anionic phospholipids were removed; a mixture of dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG) (9:1, mol:mol); and DPPC alone. The initial reduction in surface tension depended strongly on the anionic phospholipids and the subphase concentration. The acyl groups had no effect. Adsorption beyond the initial stage depended more on the mixed acyl groups, became increasingly independent of subphase concentration, and was determined instead by the interfacial concentration of the surface film. The different constituents produced the same effects in vesicles adsorbing to a clean interface or in a preexisting film to which vesicles of SP:DPPC adsorbed. Adsorption for vesicles of SP:PPL adsorbing to DPPC or of SP:DPPC to PPL above a certain threshold surface concentration followed exactly the same isotherm. Our results fit best with a two-step model for adsorption. The anionic phospholipids first promote the initial juxtaposition of vesicles to the interface. Compounds with mixed acyl constituents at the point of contact between vesicle and interface then facilitate fusion with the surface.

## INTRODUCTION

Rapid adsorption of pulmonary surfactant to the air-liquid interface is essential for normal breathing. Pulmonary surfactant is the complex mixture of lipids and proteins that forms a film on the thin liquid layer that lines the alveolar air spaces. When compressed by the decreasing surface area during exhalation, the surfactant films achieve extremely high densities, minimizing the surface tension of the air-liquid interface and the tendency of small alveoli to collapse (Schürch, 1982). The characteristics of the interfacial film itself, however, are insufficient to predict surfactant function in the lung. The most prevalent constituent of surfactant, dipalmitoyl phosphatidylcholine (DPPC), reduces surface tension quite effectively when spread artificially at an air-liquid interface and compressed in vitro. This compound functions poorly in the lung when used alone, apparently because it adsorbs slowly to the air-liquid interface (e.g., Hall et al., 1992). DPPC lacks the ability of pulmonary surfactant to restore the mechanical characteristics of an excised lung depleted of surfactant by repeated lavage (Hall et al., 1992), and is ineffective as a therapeutic agent for surfactant-deficient premature infants (Chu et al., 1967). The ability of constituents other than DPPC to facilitate rapid formation of the interfacial film is critical for surfactant function in the lung.

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These studies seek to determine the mechanisms by which surfactant vesicles adsorb to form the interfacial film. Adsorption should occur by sequential steps, the details of which remain uncertain. Previously proposed models have generally included initial diffusion of the vesicles to the interface, followed by the subsequent unraveling of the vesicles to form the interfacial film (King and Clements, 1972; Davies et al., 1986; Ivanova et al., 1989). These processes should have distinct dependence on concentration. Diffusion should depend on concentration in the subphase. Insertion into the interfacial film should instead depend on the surface concentration of the monolayer. Different phospholipids might also distinguish different processes in adsorption. In addition to DPPC, pulmonary surfactant also contains a complex mixture of other phospholipids. Although these compounds are less important than the hydrophobic surfactant proteins, they also promote adsorption (Wang et al., 1996). Ten percent of the phospholipids contain anionic headgroups, and ~65% contain unpaired acyl residues (Kahn et al., 1995). Because of the different physical characteristics contributed to the complete molecule, the anionic headgroups and mixed acyl chains seem likely to promote adsorption by different mechanisms.

We have used the effects of both concentration and phospholipid composition to distinguish different steps in surfactant adsorption. We varied both concentration and composition for vesicles in the subphase and for the interfacial film to which they adsorb. Our experiments used four different preparations of phospholipids in which the anionic headgroup and the mixed acyl chains varied independently. To enhance the physiological relevance of our studies, our

preparations contain the full biological complexity of phospholipid mixtures where possible and the native mix of hydrophobic surfactant proteins in all cases. Our results fit with a single two-step model in which the anionic phospholipids control the initial juxtaposition of vesicles to the surface, and the mixed acyl residues promote the subsequent fusion with the interface.

## MATERIALS AND METHODS

### Materials

The following phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further characterization or purification: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC); 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG).

Extracts of calf surfactant (calf lung surfactant extract, CLSE) were provided by Dr. Edmund Egan of ONY, Inc. (Amherst, NY). Calf surfactant is obtained by combining fluid lavaged from multiple calf lungs, spinning the lavaged fluid at low speed (250 $\times$ *g* for 10 min) to remove cells, and then collecting the large surfactant particles by higher speed centrifugation (12,500 $\times$ *g* for 30 min). Extraction of the resuspended particles (Bligh and Dyer, 1959) provides CLSE (Notter et al., 1983). Prior analysis has shown that the lipid in extracted calf surfactant is ~92% (mol:mol) phospholipid and 8% neutral lipid, and that the extracted surfactant also contains ~1.5% (w:w) of the hydrophobic proteins SP-B and SP-C (Kendig et al., 1989). Phosphatidylcholines (PCs) constitute most of the phospholipids (82% mol:mol), but ~10% are the anionic phosphatidylglycerol (PG) and phosphatidylinositol (PI) (Hall et al., 1994). Four major compounds dominate the PCs: 40% (of PC) DPPC; 18% palmitoyl-palmitoleoyl PC; 14% palmitoyl-oleoyl PC; and 12% palmitoyl-myristoyl PC (Kahn et al., 1995). To the best of our knowledge, no analysis of the anionic molecular species in calf surfactant has yet been published. Results from other species and our preliminary results with mass spectroscopy suggest that DPPG represents a fraction of total PG similar to the ratio for DPPC/PC, but that the content of dipalmitoyl PI is quite small (Schlame et al., 1986, 1988; Hayashi et al., 1990) .

Mixtures of DPPC:DPPG (DPPC/G) were combined at the approximate ratio of the zwitterionic to anionic phospholipid headgroups (9:1, mol:mol) present in calf surfactant. Preparations containing the complete set of purified phospholipids (PPL), the modified PPL (mPPL) from which the anionic phospholipids were removed, and the hydrophobic surfactant proteins SP-B and SP-C (SP) were obtained from CLSE by column chromatography. The proteins, phospholipids, and neutral lipids separate into distinct peaks on gel permeation chromatography through LH-20 using acidified chloroform:methanol (Takahashi and Fujiwara, 1986). Collection of the phospholipid fractions provides the PPL (Hall et al., 1994). Using the same solvent without acid, the anionic phospholipids PG and PI stick to the column. mPPL and SP were obtained using the neutral solvent (Hall et al., 1994) .

### Methods

#### *Biochemical assays*

Phospholipid concentrations were determined by phosphate assay (Ames, 1966). Proteins were assayed with amido black on material precipitated with trichloroacetic acid (Kaplan and Pedersen, 1989) .

#### *Dispersion of samples*

Samples of proteins and lipids were combined in chloroform at 0.122 $\mu$g protein/$\mu$mol phospholipid to replicate the ratio in CLSE (Hall et al., 1994). After evaporation of the solvent, the mixture was suspended at 1.95 mM phospholipid in 150 mM NaCl, 1.5 mM $CaCl_2$, 10 mM Hepes pH 7.0 (HSC) by sonication at ambient temperature. Initial bath sonication (Branson, Danbury, CT) for 2–3 min removed material from the sides of the vial. Subsequent probe sonication (Virtis, Gardiner, NY) at 5 W achieved more uniform preparations. Probe sonication for 15-s intervals were repeated until the optical density at 320 nm reached 1.8.

#### *Measurements of adsorption*

*Wilhelmy plate.* The kinetics of surfactant adsorption were monitored by measuring the reduction in surface tension produced by the formation of the surface film after the injection of surfactant vesicles below an air-liquid interface (King and Clements, 1972). Our apparatus used a Wilhelmy plate to measure surface tension at the air-water interface of 30 ml HSC contained within a Teflon cup stirred with a magnetic bar and maintained at 37°C. Suspensions of lipid and protein were injected into the bulk liquid via tubing inserted through the cup wall. The force on the 2-cm Wilhelmy plate composed of filter paper was measured by a home-built force transducer consisting of a flat spring connected to a displacement transducer (Omega Engineering, Stamford, CT). The signal was amplified by custom-built electronics and processed through an analog-to-digital converter (NB-A2000, National Instruments, Austin, Texas) to a personal computer (Macintosh Quadra 650, Apple Inc., Cupertino, CA) where it was averaged and filtered using software developed through a graphical programming interface (LabVIEW, National Instruments). The program scanned at a rate of 500 Hz, filtered readings more than two standard deviations from the mean of 100 scans, and box-car averaged over every 500 scans. Calibration of the force transducer with standard weights confirmed the linear response over the range used in our experiments. The shift in baseline caused by buoyancy on the plate was removed by setting the signal to 70 mN/m for the clean interface at the beginning of each experiment.

Experiments with spread films used appropriate volumes of 67 $\mu$M phospholipid in hexane-ethanol (9:1, v:v) added in droplets to the clean interface. An interval of at least 10 min allowed for evaporation of solvent before subsequent measurements of surface tension. Experiments concerning the dependence of surface tension on surface concentration at equilibrium used sequential 15-$\mu$l aliquots of the phospholipid solution to vary surface concentration. Studies concerning the effect of preexisting films on adsorption recorded the surface tension of the spread film for 70 s before injection of the surfactant vesicles into the subphase. All data represent the average of three experiments unless indicated otherwise with error bars giving $\pm$ SD only at selected points to maximize clarity of presentation.

*Bubble surfactometer*. For experiments at 1 mM phospholipid, measurements of adsorption used a Pulsating Bubble Surfactometer (PBS; Electronetics, Amherst, NY) (Enhorning, 1977) to minimize the amount of material required at that higher concentration. This instrument determined the surface tension at 37°C of an air bubble formed on the tip of a capillary tube in a dispersion of surfactant. Measurements of the hydrostatic pressure difference across the air-liquid interface provide the basis for calculating

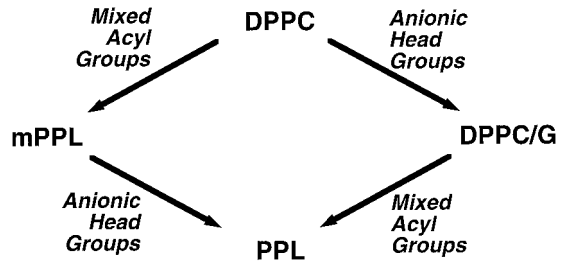


FIGURE 1 Relationship among the different preparations of phospholipids used in these studies. Arrows indicate which pairs of constituents differ only by the content of anionic headgroups or of mixed acyl chains.

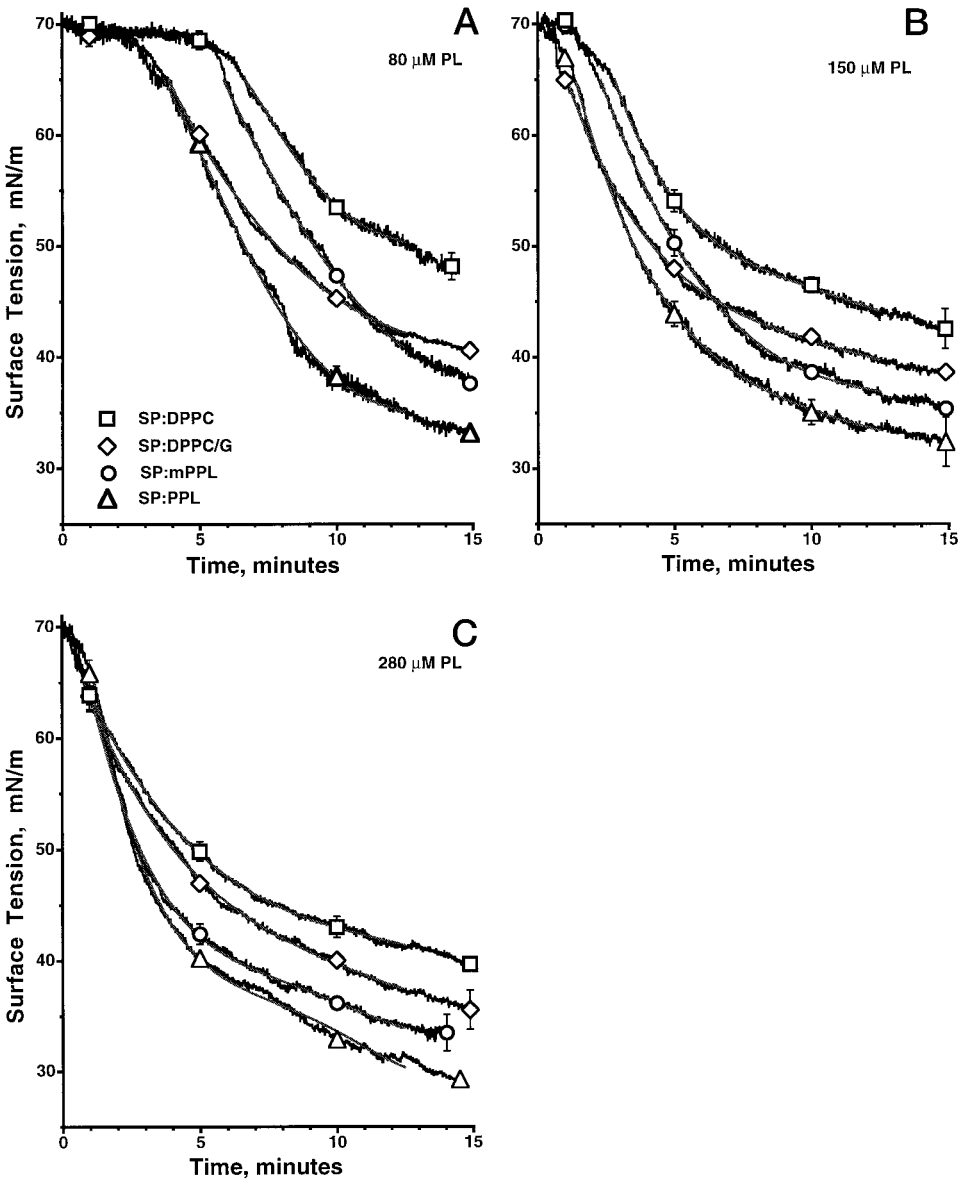


FIGURE 2 Effect of phospholipid composition on adsorption of vesicles at three different subphase concentrations. Surface tension was monitored after injection of vesicles into a subphase of HSC at 37°C to the following final concentrations: (*A*) 80 $\mu$M; (*B*) 150 $\mu$M; (*C*) 280 $\mu$M. Vesicles contained the hydrophobic surfactant proteins mixed with the different phospholipid preparations indicated in the legend common to all three panels. Data are mean of three experiments with SD shown only at selected points for clarity of presentation. Superimposed gray curves are fourth-order polynomials fit to averaged data for surface tension $<65$ mN/m and plotted to 12.5 min following injection.

surface tension at known radii using the law of Young and Laplace (Enhorning, 1977). Surface tension was recorded as 70 mN/m at zero time, and then measured at specific intervals after formation of the bubble. Experiments were performed five times except for samples of SP:DPPC/G for which $n = 10$.

## RESULTS

### Phospholipid preparations

Our studies used mixtures of the hydrophobic surfactant proteins with four different sets of phospholipids (Fig. 1). These ranged in complexity from DPPC alone, with no compounds containing anionic headgroups or mixed acyl chains present, to the full set of purified phospholipids (PPL) obtained from calf surfactant, which included the full complement of both sets of constituents. DPPC mixed with DPPG (DPPC/G) achieved the approximate ratio of zwitterionic to anionic headgroups (9:1 mol:mol) found in calf surfactant, but contained no compounds with mixed acyl chains. The modified PPL (mPPL), obtained by removing the anionic phospholipids from PPL, provided the complementary preparation with the full mixture of acyl chains but no anionic headgroups. The final surface tension achieved by these mixtures at equilibrium was unaffected by composition. After 3 h, SP:DPPC and SP:PPL at 150 $\mu$M phospholipid adsorbed to stable surface tensions of $22.2 \pm 0.9$ and $21.6 \pm 0.4$ mN/m, respectively. These four preparations therefore provide a set of mixtures that independently varied the content of anionic headgroups and mixed acyl residues (Fig. 1), and that affected the rate of adsorption but not the ultimate surface tension achieved.

## Adsorption to a clean interface

Models of surfactant adsorption commonly include sequential steps that should differ in their dependence on concentration both of vesicles in the subphase and of the surface film at the interface, and which might have different dependence on composition. Our approach therefore was to determine the effect on adsorption rates of varying the concentration and phospholipid composition both in the subphase and at the interface. Initial studies considered adsorption to a clean interface, with variation only of the subphase vesicles.

Subphase concentration produced its greatest effect during the earliest stages of adsorption. At lower concentrations, surface tension fell only after an initial delay (Fig. 2). For each preparation, both the extent of the delay and the rate of the initial fall in surface tension depended on subphase concentration (Fig. 2). For SP:DPPC, for instance, the lag time to reach 68 mN/m was 5.3 ± 0.7 min at 80 $\mu$M phospholipid, and 1.8 ± 0.3 min at 150 $\mu$M. The delay was undetectably small at 280 $\mu$M. Concentration had similar effects on the initial rate at which surface tension fell after completion of the lag phase. To obtain the slopes of the isotherms, we used fourth-order polynomials, which fit the data well between 68 mN/m and 12.5 min (Fig. 2), and calculated the first derivatives. In the early stages of adsorption before surface tension fell appreciably, higher concentration produced steeper slopes for each of the four preparations (Fig. 3).

Comparison between the different preparations showed that the initial stage of adsorption also depended on composition. At any given concentration, the presence or absence of anionic phospholipids determined the extent of the delay. For 80 $\mu$M phospholipid, the preparations without anionic headgroups, SP:DPPC and SP:mPPL, reached 68 mN/m in 5.3 ± 0.7 and 5.3 ± 0.3 min, respectively. The vesicles with anionic phospholipids, SP:DPPC/G and SP:PPL, required the shorter times of 2.8 ± 0.2 and 2.8 ± 0.1 min. This effect of composition persisted at 150 $\mu$M phospholipid, where SP:DPPC and SP:mPPL required 1.8 ± 0.3 and 1.5 ± 0.2 min to reach 68 mN/m, and the mixtures with DPPC/G and PPL with anionic lipids both eliminated any detectable delay. The initial reduction in surface tension

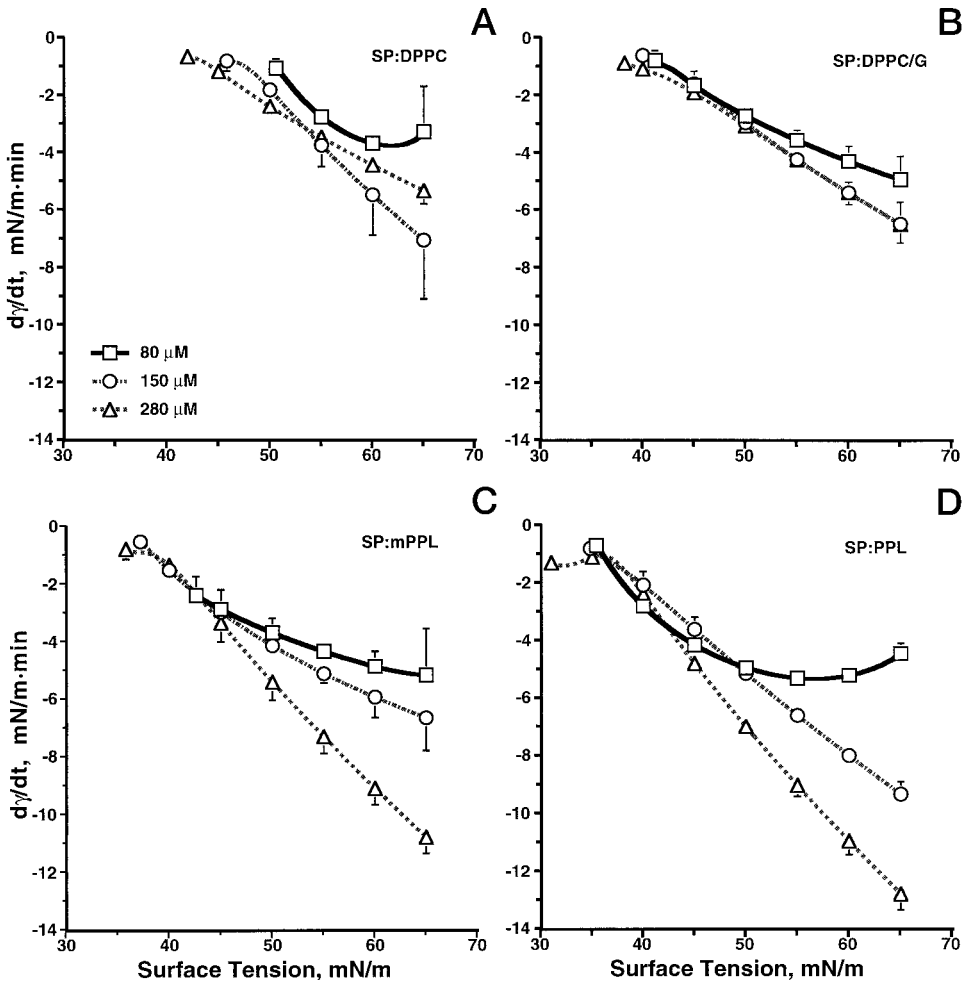


FIGURE 3 Effect of subphase concentration on rate of adsorption. Adsorbing vesicles contained the hydrophobic surfactant proteins mixed with the following phospholipid preparations: (*A*) DPPC; (*B*) DPPC/G; (*C*) mPPL; (*D*) PPL. Curves give the average value of the derivative (dγ/dt) calculated from fourth-order polynomials fitted to individual adsorption isotherms of surface tension (γ) as a function of time. Symbols label selected points only to distinguish the different curves. Mean ± SD, $n = 3$.

therefore occurred more quickly at higher concentrations and with vesicles containing anionic phospholipids. The composition of the acyl groups had no effect.

The dependence of kinetics on both subphase concentration and composition changed as surface tension fell. After an initial reduction in surface tension, the isotherms at the different subphase concentrations all produced roughly parallel isotherms for any given preparation (Fig. 2), suggesting that the subphase concentration was less important at this later stage of adsorption. The slopes of the curves at the different concentrations converged as surface tension fell, producing common values for each preparation that were independent of subphase concentration at surface tensions below 45–50 mN/m (Fig. 3). The initial and later stages of adsorption therefore had distinct dependence on subphase concentration.

Studies with the different preparations showed that the kinetics of early and late adsorption also had distinct dependence on phospholipid composition. In contrast to the earlier stage, during which only the anionic headgroups had an effect, the slopes (Fig. 3) taken from the isotherms (Fig. 2) at lower surface tensions showed that both the anionic headgroups and the mixed acyl residues promoted faster adsorption. The mixed acyl groups, however, produced the dominant effect. Consequently, for each subphase concentration the rates at all surface tensions $<60$ mN/m increased in the order DPPC $<$ DPPC/G $<$ mPPL $<$ PPL (Fig. 3). The effect of the acyl groups was most evident from the isotherms for 80 $\mu$M and 150 $\mu$M phospholipid (Fig. 2, *A* and *B*), at which SP:mPPL, which lacked anionic headgroups and therefore started reducing surface tension later than SP:DPPC/G, nonetheless achieved lower values by the end of the 15-min experiment. The effect of the anionic groups was most apparent from the experiments at 280 $\mu$M phospholipid (Fig. 2 *C*). Despite the similar initial slopes at this higher concentration, the isotherms for the preparations with the same acyl groups (SP:DPPC and SP:DPPC/G; SP:mPPL and SP:PPL) did separate at later times, with the presence of the anionic headgroups producing lower surface tensions (PPL $<$ mPPL; DPPC/G $<$ DPPC). The predominant influence of the acyl residues with the headgroups having a smaller effect distinguished this later stage from initial adsorption.

We extended the compositional studies to 1 mM phospholipid to determine which part of the molecule would dominate later adsorption at this higher concentration (Fig. 4). These experiments used a PBS rather than the Wilhelmy plate method. The PBS required much smaller subphase volumes (25 $\mu$l) than the standard instrument (30 ml) and avoided the requirement for prohibitively large amounts of material at this higher concentration. The adsorption isotherms depended only on the content of mixed acyl residues; the anionic headgroups had no effect (Fig. 4). Adsorption for SP:DPPC and SP:DPPC/G, with the paired palmitoyl residues but different headgroup compositions,

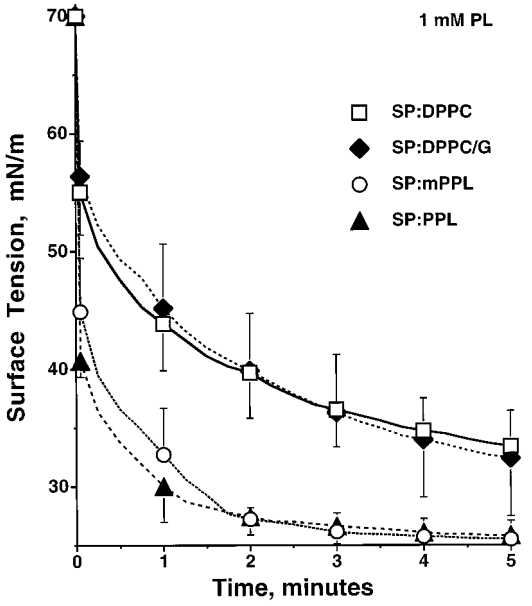


FIGURE 4 Effect of composition on adsorption at 1 mM phospholipid. A Pulsating Bubble Surfactometer with pulsation rate set to zero measured the surface tension. Values were recorded as 70 mN/m at time 0, and then measured at 1 mM phospholipid and 37°C as a function of time after formation of the bubble in dispersions of the indicated preparations. Data are mean values for five experiments for all preparations except SP:DPPC/G, for which $n = 10$. SD values are shown only at selected points.

followed the same curve. SP:mPPL and SP:PPL, which contain the same mixed acyl residues but different headgroups, both followed the same isotherm at surface tensions significantly below those for SP:DPPC and SP:DPPC/G (Fig. 4). The results with the PBS fit with the trends established on the standard instrument that anionic groups predominantly affected the concentration-dependent portion of adsorption, and that their effect became inapparent during rapid initial adsorption at higher concentration. The effect of the acyl residues was independent of concentration, and persisted to higher concentrations.

## Adsorption to a preexisting film

During adsorption to an initially clean surface, the constituents of the vesicles quickly occupy the interface. The effect of any component could then occur either in the vesicle or in the film. We therefore also conducted a set of experiments in which we measured adsorption to a preexisting monolayer with variable composition to distinguish effects in vesicles from those in the surface film. We injected vesicles of SP:DPPC below spread monolayers containing the different phospholipids. Fortunately, the surface tension of the monolayers showed minimal variation with composition (Fig. 5). Films containing DPPC and PPL, with or without the surfactant proteins, had equivalent relationships between surface concentration and surface tension (Fig. 5).

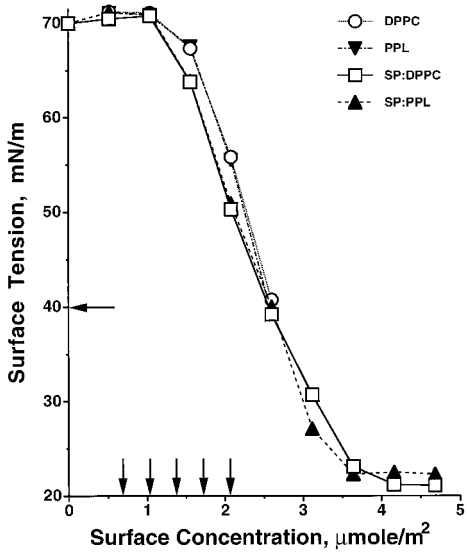


FIGURE 5 Variation of surface tension for spread films containing DPPC and PPL with or without the hydrophobic surfactant proteins. Sequential aliquots of samples in hexane:ethanol (9:1, v:v) were spread at the surface of a subphase containing HSC to the desired surface concentration. Samples containing the protein:phospholipid mixtures achieved a stable surface tension after 10 min at 37°C to allow for solvent evaporation. Samples containing only the phospholipids required increasingly long periods for surface tension to stabilize at higher concentrations, and consequently no measurements were made without proteins above 2.6 $\mu$mol/m$^2$. Data are plotted as mean ± SD, although error bars are smaller than the size of the symbols. Arrows indicate the conditions to which films were spread in subsequent experiments concerning adsorption to preexisting films.

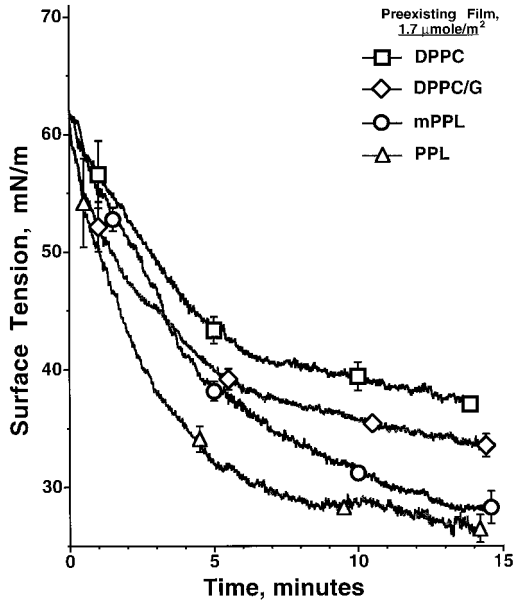


FIGURE 6 Effect of composition in preexisting films on the adsorption of SP:DPPC. Vesicles of SP:DPPC were injected into the subphase to a final concentration of 150 $\mu$M below prespread films containing the indicated phospholipids with a total surface concentration of 1.7 $\mu$mol/m$^2$. Data are mean values for three experiments with SD shown only at selected points.

Adsorption to films with different compositions but the same surface concentration therefore started at similar surface tensions, and allowed direct comparison of the effect of different surface compositions on the adsorption isotherms.

The different phospholipids in interfacial films showed distinct effects on the adsorption of SP:DPPC (Fig. 6). Anionic phospholipids again accelerated the initial fall in surface tension and determined the initial phase of adsorption. Adsorption to monolayers containing DPPC/G and PPL with the anionic phospholipids followed the same curve during the first minute after injection of the DPPC vesicles (Fig. 6). For films of DPPC and mPPL without anionic lipids surface tension fell more slowly, but again the two preparations shared the same initial isotherm despite their different acyl chains (Fig. 6). At later times, the mixed acyl groups at the interface asserted a greater effect, just as they did in vesicles adsorbing to a clean interface. Despite the slower start, surface tension for adsorption to mPPL fell below values for DPPC/G at 3.5 min and approached values for PPL by the end of the 15-min experiment (Fig. 6). In contrast, the curve for adsorption to DPPC/G without mixed acyl groups became parallel to the isotherm for DPPC despite an initially faster reduction in surface tension (Fig. 6). These experiments used a standard subphase concentration for the SP:DPPC of 150 $\mu$M phospholipid and a surface concentration of 1.7 $\mu$mol/m$^2$ for the preexisting films. Under these conditions the anionic headgroups and the mixed acyl residues in the interfacial film had the same effect on adsorption as when present in the vesicles.

We also varied the surface concentration of the interfacial film to determine if the beneficial effect of the mixed lipids on adsorption of SP:DPPC changed with the density of the film. Studies compared adsorption of 150 $\mu$M SP:DPPC to preexisting films of either DPPC or PPL at different surface concentrations, and included parallel experiments with SP:PPL to serve as controls (Fig. 7). For vesicles containing SP:PPL, the composition of the monolayer was unimportant. Surface tension fell along the same curves for adsorption to DPPC or PPL at all surface concentrations. For vesicles of SP:DPPC, however, the composition of the monolayer altered adsorption, although in a manner that depended on surface concentration. With films at 0.7 $\mu$mol/m$^2$ (not shown) and 1.0 $\mu$mol/m$^2$ (Fig. 7 *A*), composition had no effect, and SP:DPPC at 150 $\mu$M adsorbed to DPPC and to PPL along equivalent isotherms. With both films, vesicles of SP:DPPC lowered surface tension more slowly than SP:PPL. At 1.4 $\mu$mol/m$^2$, PPL at the interface significantly accelerated adsorption of the SP:DPPC vesicles, producing an isotherm that fell approximately midway between the curves for SP:DPPC to DPPC and for SP:PPL (Fig. 7 *B*). At 1.7 $\mu$mol/m$^2$ (Fig. 7 *C*) and 2.1 $\mu$mol/m$^2$ (data

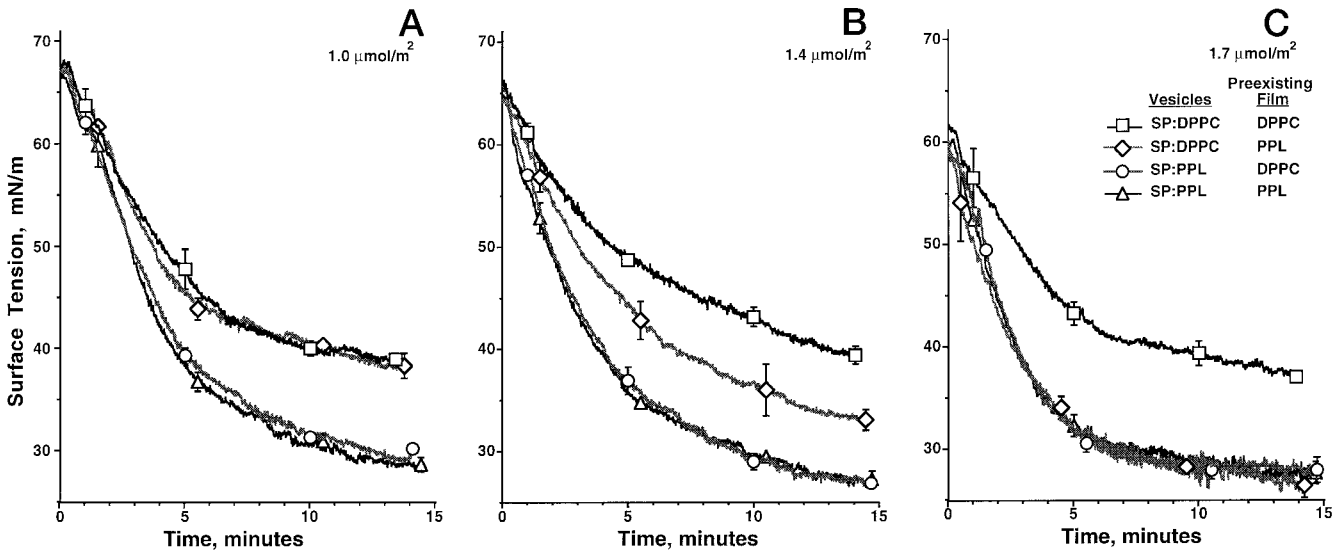


FIGURE 7 Effect of surface concentration on adsorption to a preexisting film. Particles of SP:DPPC or SP:PPL were injected into the subphase to a final concentration of 150 $\mu$M phospholipid below films of DPPC or PPL prespread to the following surface concentrations: (*A*) 1.0 $\mu$mol/m$^2$; (*B*) 1.4 $\mu$mol/m$^2$; and (*C*) 1.7 $\mu$mol/m$^2$. Data are mean values of surface tension as a function of time following injection of the surfactant preparations with SD, shown only at selected points. $n = 3$ experiments.

not shown), adsorption of SP:DPPC to PPL followed the same curve as for SP:PPL to PPL. Above a threshold surface concentration of 1.4 $\mu$mol/m$^2$, the mixed phospholipids therefore produced exactly the same acceleration of adsorption relative to DPPC whether present in the vesicles or in the interfacial film.

We also investigated the extent to which DPPC could substitute for PPL in providing this threshold surface concentration. We reasoned that 1.4 $\mu$mol/m$^2$ might represent a requirement not simply for that amount of PPL, but rather for a film with that total surface concentration, only part of which must be PPL. The effectiveness of the preexisting monolayer would then be preserved if other material such as DPPC replaced part of the film, as long as the total surface concentration remained above the threshold value. DPPC, however, was unable to substitute for part of the PPL film (Fig. 8). PPL at 1.0 $\mu$mol/m$^2$ mixed with DPPC to achieve total surface concentrations of 1.7 and 2.1 $\mu$mol/m$^2$ altered only the first few minutes of adsorption (Fig. 8). The threshold requirement for 1.4 $\mu$mol/m$^2$ PPL at the interface appeared to be unaffected by the presence of additional DPPC.

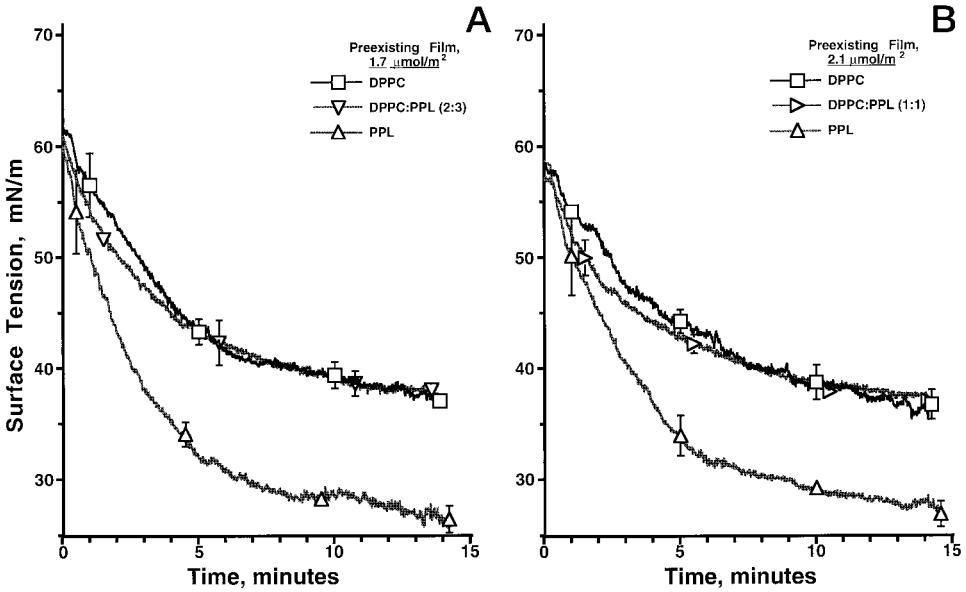


FIGURE 8 Effect of preexisting films containing DPPC and PPL on the adsorption of SP:DPPC. Vesicles of SP:DPPC were injected into the subphase to a final concentration of 150 $\mu$M below prespread films containing DPPC, PPL, or mixtures of DPPC and PPL at the ratios (mol:mol) indicated. The prespread films had the following total surface concentrations: (*A*) 1.7 $\mu$mol/m$^2$; (*B*) 2.1 $\mu$mol/m$^2$. Data are mean of three experiments, with SD shown only at selected points.

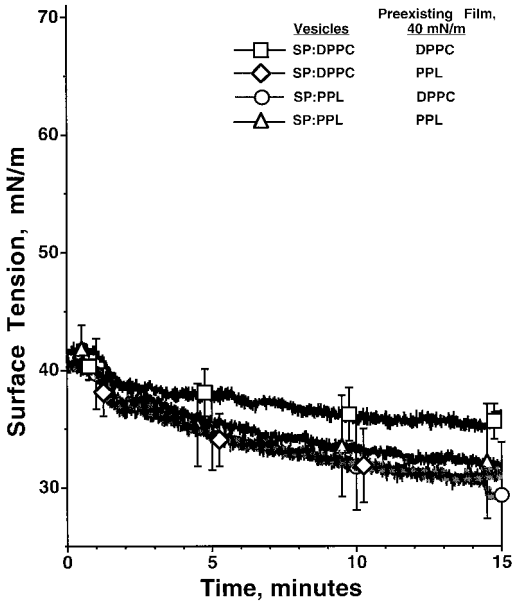


FIGURE 9 Adsorption to a preexisting film with a high surface concentration. Vesicles of SP:DPPC or SP:PPL were injected into the subphase to a final concentration of 150 $\mu$M below preexisting films containing either DPPC or PPL added to achieve a surface tension of 40 mN/m. Data are mean of three experiments with SD shown only at selected points.

Final experiments determined if DPPC at the interface might hinder adsorption when present in sufficiently high surface concentrations. If DPPC forms films that are less fluid than for PPL, it might limit rearrangements within the surface film to accommodate adsorbing material. Vesicles of SP:PPL, however, produced equivalent isotherms with films of DPPC and PPL at all surface concentrations studied (Figs. 8 and 10). Films spread to 40 mN/m represented an approximate upper limit on the surface concentration that would still allow adsorbing vesicles to produce an easily measurable change in surface tension. Even at that level, adsorption of 150 $\mu$M SP:PPL to films of DPPC or to PPL was similar (Fig. 9). These results indicated that for vesicles containing PPL, high surface concentrations of DPPC were unable to impede adsorption.

## DISCUSSION

Our results suggest that the adsorption of pulmonary surfactant to an air-liquid interface can be considered in terms of two sequential steps. These discrete processes are distinguished by their different dependence on phospholipid composition and on concentration both in the subphase and at the surface. These different characteristics allow the discrimination of the two steps and suggest possible mechanisms for each process (Fig. 10).

The concentration dependence of the adsorption rate suggests that the rate-limiting step changes as the process proceeds. Initially, when the high surface tension indicates

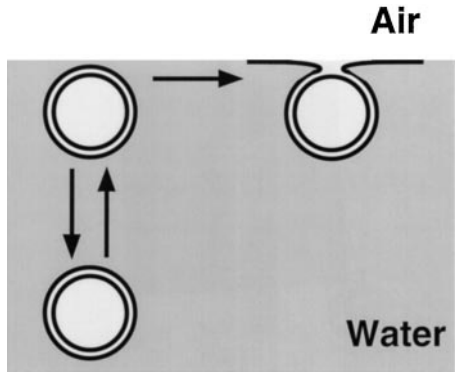


FIGURE 10 Two-step model for adsorption of surfactant vesicles to form an interfacial film. Vesicles approach the interface in the first step and then fuse with the interface in the second step.

relatively little material at the interface, the rate depends on the concentration in the subphase. This suggests a process that begins in the subphase, suggesting that transport to the interface might be the crucial early step. Later, when lower surface tensions indicate that the adsorbed film has achieved a significant surface concentration, the dependence shifts. For each preparation, adsorption rates at different concentrations approach each other. The rates vary only with surface tension and are unaffected by subphase concentration, suggesting a process that depends only on material at the surface. The convergence of the rates for different subphase concentrations occurs while surface tension continues to decrease at levels well above equilibrium surface pressures. Consequently, the trivial observation that the rates must become independent of concentration at equilibrium when they all reach zero does not explain our results. Instead, the dependence on concentration only at the surface and not in the subphase suggests that a process localized to the air-water interface limits the rate during this later stage of adsorption.

The compositional dependence provides further insight into mechanisms involved in each stage. The initial process varies only with the content of anionic lipids. Both the initial lag-time before surface tension begins to fall and the rate at which surface tension first decreases depend on the anionic headgroups. We have no means of following adsorption during the lag phase, but we speculate that the delay represents only the time during which material accumulates more or less continuously until surface concentration reaches a level sufficient to affect surface tension. The anionic lipids promote faster adsorption whether present in the bilayer vesicles or in the surface monolayer. Since simple diffusion of the vesicles seems unlikely to vary greatly with their charge, this early stage probably represents some process other than simple transport across an unstirred layer.

The effects of the anionic phospholipids suggest that electrostatic interactions reduce a barrier to adsorption en-

countered by neutral vesicles. Surfactant adsorption would then be analogous to the initial steps in the fusion of two bilayers. The thermodynamic barrier that opposes the close approach of neutral bilayers is well-documented (Leikin et al., 1993; McIntosh and Simon, 1994), and consists of some combination of hydration forces and entropic effects (McIntosh et al., 1995; Israelachvili and Wennerstrom, 1996). The same factors should also oppose the juxtaposition of a surfactant vesicle to an air-liquid interface. For two bilayers, electrostatic interactions tend to counteract this barrier and permit the initial contact between vesicles. Calcium binds two anionic phospholipids (Feigenson, 1986), and if these compounds occur in *trans* configuration on opposing bilayers (Portis et al., 1979), the salt bridge across the gap between two vesicles would promote their close approximation. For adsorption, however, the anionic phospholipids accelerate adsorption by interactions for which such *trans* salt bridges are impossible. Anionic compounds facilitate both adsorption of the different preparations to a clean interface (Fig. 2) and of SP:DPPC to preexisting films (Fig. 6). In both cases, the anionic lipids exist on only one side of the gap between interface and vesicle. In prior studies with electrolyte, $Na^+$ was just as effective as $Ca^{2+}$ in promoting this early stage of adsorption, again arguing against the bridging of a cation between two anionic lipids (Oosterlaken-Dijksterhuis et al., 1991a). Electrolyte was essential in these prior studies, and vesicles of SP-B:DPPC:egg PG (1:7:3) had no effect on surface tension until electrolyte was added (Oosterlaken-Dijksterhuis et al., 1991a). Our results suggest either that the close approach of bilayers does not involve salt bridges between the two vesicles, or that the details for this early stage of adsorption differ from the initial steps in bilayer fusion. Our findings generally fit, however, with a model in which electrostatic interactions involving the anionic lipids minimize the barrier that limits the close approach of vesicles to the surface and facilitate their juxtaposition to the air-liquid interface.

Just as the concentration dependence changes when surface tension falls, so the mixed acyl residues quickly surpass the anionic headgroups in their ability to promote adsorption. As with the earlier effect of the anionic compounds, the mixed acyl groups have similar effects whether present in the bilayer vesicle or in the surface monolayer. Studies using all four preparations demonstrate the ability of the compounds with mixed acyl groups to promote adsorption in either location (Figs. 2 and 7). The experiments directly comparing PPL with DPPC (Fig. 7), however, are perhaps most informative. For monolayers above a threshold surface concentration, PPL accelerates adsorption of vesicles to the monolayer to the same extent whether present in the vesicles, the monolayer, or both (Fig. 7). Surface tension falls along exactly the same curve for all three configurations (Fig. 7). The equivalence of these isotherms argues that the lipids must be important for a structure to which components from vesicle and monolayer have equal access. The high energy intermediate for surfactant adsorption is then likely to be the initial complex formed at the point of first contact between vesicle and interface. Such a structure would be analogous to the highly curved intermediate hypothesized to be rate-limiting in the fusion of two bilayers (Chernomordik et al., 1995). The mixed acyl groups should produce more fluid structures that would more readily permit such a highly curved intermediate. The surfactant proteins have similar ability to accelerate adsorption when present in monolayers as well as in bilayer vesicles (Oosterlaken-Dijksterhuis et al., 1991a, b), which argues that the proteins also would promote or stabilize such a complex.

The equivalent effects of PPL in the vesicles and in the monolayer specifically argue against a significant limitation of adsorption by the rate of spreading across the interface. Effects distant from the site of vesicle insertion, such as spreading or rearrangement of constituents to accommodate new material, would depend on the composition of the monolayer. DPPC, with its expected greater viscosity, should spread and rearrange more slowly than the more fluid phospholipids with mixed acyl chains. Preexisting monolayers of DPPC, however, with surface pressures as high as 30 mN/m, have no effect on the adsorption of SP:PPL (Fig. 9). Movement in the film away from the site of insertion therefore seems less likely to be a major determinant of the rate of adsorption.

The ability of the preexisting monolayer to enhance the rate of adsorption for SP:DPPC has an interesting requirement for both composition and surface concentration. Films of PPL accelerate adsorption only if the surface concentration exceeds roughly 1.4 $\mu$mol/m$^2$. This level corresponds to the approximate point at which the PPL monolayer first begins to lower surface tension, indicating conversion of the two-dimensional film from the gas to the liquid-expanded (LE) phase. The different effects on adsorption caused by increasing the preexisting monolayer from 1.0 to 1.4 to 1.7 $\mu$mol/m$^2$ might then reflect the presence of more LE phase when moving through the gas-LE coexistence region in the phase diagram. The dependence on composition, however, makes it unlikely that the enhanced adsorption requires only a preexisting LE monolayer. Films of DPPC at 37°C remain homogeneously LE to surface concentrations well above 2 $\mu$mol/m$^2$ (Albrecht et al., 1978; Discher et al., 1996), and so at the concentrations for which the PPL monolayers enhanced adsorption, both DPPC and PPL would occur at the interface as the LE phase. The beneficial effect produced by the preexisting films does seem to reflect a requirement for specific amounts of PPL.

Our results then provide solid supporting evidence that has previously been lacking for the widely considered (King and Clements, 1972; Davies et al., 1986; Ivanova et al., 1989) two-step model of adsorption consisting of transport of surfactant vesicles to the interface followed by unraveling to form the surface film (Fig. 10). Our results also suggest more specific characteristics of the mechanisms

involved in each step. The initial juxtaposition of the vesicles to the surface is facilitated by electrostatic interactions involving the anionic phospholipids. Compounds with mixed acyl groups promote the subsequent fusion of vesicles with the interface, apparently by stabilizing the initial complex between bilayer vesicle and interfacial monolayer.

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