Lipid compositional analysis of pulmonary surfactant monolayers and monolayer-associated reservoirs

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Abstract **Pulmonary surfactant is a lipid:protein complex containing dipalmitoyl-phosphatidylcholine (DPPC) as the major component. Recent studies indicate adsorbed surfactant films consist of a surface monolayer and a monolayer-associated reservoir. It has been hypothesized that the monolayer and its functionally contiguous reservoir may be enriched in DPPC relative to bulk phase surfactant. We investigated the compositional relationship between the monolayer and its reservoir using paper-supported wet bridges to transfer films from adsorbing dishes to clean surfaces on spreading dishes. Spreading films appear to form monolayers in the spreading dishes. We employed bovine lipid extract surfactant [BLES(chol)] containing [^{3}H]DPPC and either [^{14}C]palmitoyl, oleoyl-phosphatidylcholine (POPC), [^{14}C]dipalmitoyl-phosphatidylglycerol (DPPG), [^{14}C]palmitoyl, oleoyl-phosphatidylglycerol (POPG), or [14C]cholesterol. Radiolabeled phosphatidylglycerols were prepared using phospholipase D. The studies demonstrated that the [^{3}H]DPPC-[^{14}C] POPC ratios were the same in the prepared BLES dispersions as in Langmuir-Blodgett films, indicating a lack of DPPC selectivity during film formation. Furthermore, identical ^{3}H-^{14}C isotopic ratios were observed with DPPC and either ^{14}C-labeled POPC, DPPG, POPG, or cholesterol in the original dispersions, the bulk phases in adsorption dish D1, and monolayers recovered from spreading dish D2. These relationships remained unperturbed with 2-fold increases in bulk concentrations in D1 and 10-fold variations in D1-D2 surface area. These results indicate adsorbed surfactant monolayers and their associated reservoirs possess similar lipid compositions and argue against selective adsorption of DPPC.**—Yu, S-H., and F. Possmayer. **Lipid compositional analysis of pulmonary surfactant monolayers and monolayer-associated reservoirs.** ***J. Lipid Res.*** **2003.** 44: **621–629.**

Supplementary key words bovine lipid extract surfactant • cholesterol • dipalmitoylphosphatidylcholine • Langmuir-Blodgett films • phosphatidylcholine • phosphatidylglycerol • respiratory distress syndrome • surfactant multilayers

It is generally agreed that the alveolar surface is covered by a continuous thin layer of water that supports a surface active film of pulmonary surfactant (1, 2) [for review see (3–7)]. Through its ability to reduce the surface tension of this air-water interface, pulmonary surfactant stabilizes the terminal air spaces. Considerable evidence has accumulated indicating that surfactant films are composed of more than a single monolayer. Pattle (8) first proposed that the surfactant film overlying the alveolar lining layer consists of a monomolecular layer and underlying material that serves as a reservoir. Using electron microscopy, Weibel and Gil (9) observed the presence of lamellar layers of phospholipids with three to six repeating distances of 38–51 Å on the alveolar epithelial surface of rat lungs. Studies by Manabe (2) and, more recently, Bastacky et al. (1) using scanning electron microscopic studies indicate that the alveolar lining layer is continuous and its surface contains many lipidic structures. In vitro studies involving surface films adsorbed from surfactant dispersions have also provided evidence indicating the surface monolayer is accompanied by a functional continuous reservoir (10–13). Surfactant reservoirs that can provide phospholipids to the air-water interface during surface area expansion can also be created during film compression (14–17) [as reviewed in refs. (18, 19)].

Pulmonary surfactant consists of ~90% lipids and ~10% protein. The phospholipid composition of bovine pulmonary surfactant, which is representative of mammalian species, consists of ~80% total phosphatidylcholines (PCs), 10–15% phosphatidylglycerol (PG), 2–3% each of phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin, and 1–2% lyso-*bis*- phosphatidic acid (20). Dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) are major molecular species, while dipalmitoylphosphatidylglycerol (DPPG) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) are present at significant levels (20–22). Bovine pulmonary surfactant also contains ~4% neutral lipids, of which ~90% is cholesterol. Pulmonary surfactant proteins (SPs) consist of two small hydrophobic proteins, SP-B and SP-C, and two hydrophilic complex glycoproteins, SP-A and SP-D (23–28).

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The rapid adsorption of surfactant to the air-alveolar surface to form surface-active films is essential for initiating and maintaining normal lung function (29, 30). DPPC, the main component of pulmonary surfactant, possesses a bilayer gel-to-liquid crystal transition temperature of 41°C and an overall cylindrical shape (31, 32), characteristics that favor the ability of DPPC to sustain high surface pressures of close to 70 mN/m at physiological temperatures. These properties allow spread monolayers of DPPC to reduce surface tension to low values, approaching 0 mN/m on the Langmuir-Wilhelmy surface balance, whereas spread monolayers of unsaturated phospholipids collapse near the equilibrium surface tension of ~24 mN/m (3, 5, 6, 30, 33). However, at physiological temperatures, liposomes of DPPC adsorb very slowly. Since natural and lipid extract surfactants adsorb very rapidly, the other surfactant components appear critical for adsorption of DPPC into the surface film. The ability of surfactant films to attain low surface tension approaching 0 mN/m during compression (8, 34–36) has long been attributed to the formation of a monolayer highly enriched in DPPC [as reviewed in refs. (3–6, 33, 37, 38)]. It has been suggested that DPPC enrichment during film compression arises through squeeze-out of the more fluid, non-DPPC lipids (39–41). Repeated compression-expansion cycles during breathing could result in monolayers highly enriched in DPPC by this mechanism. The presence of a surface monolayer highly enriched in DPPC is consistent with the properties of lung (42).

Recent physicochemical measurements have demonstrated that surface area reductions required to attain surface tensions near 0 mN/m during initial film compression of adsorbed surfactant films can be lower than that predicted by the DPPC content of surfactant (37, 43–46). These observations led to the proposal that the surface monolayer may become enriched in DPPC during adsorption (3–6, 37, 38).

We have previously used a filter paper-supported wet bridge technique (47, 48) to transfer lipid from the surface of adsorbing dishes containing surfactant dispersions to the surface of spreading dishes (13). The small quantities of material present in the transferred films made it difficult to measure lipid compositions accurately. Nevertheless, we obtained evidence indicating both [^{14}C]DPPC and [^{14}C]cholesterol were incorporated into the surface monolayer (13). We also obtained evidence indicating the underlying reservoir was functionally contiguous with the surface monolayer. However, the lipid compositions of the adsorbed monolayer and its associated reservoir have not been directly determined. In the present studies, we have used [^{3}H]DPPC and [^{14}C]POPC-labeled BLES(chol) to compare the relative amounts of these radiolipids in the bulk phase, the surface monolayer, and the surface-associated reservoir. In addition, [^{14}C]DPPG and [^{14}C]POPG, which are not commercially available, were prepared enzymatically and used for similar experiments. [^{3}H]DPPC and [^{14}C]cholesterol mixed with the bovine surfactant extract were also studied. Taken together, our results indicate that the [^{3}H]DPPC content of the adsorbed monolayers is not enriched relative to the surface-associated reservoir or lipid extract surfactant in the bulk phase.

MATERIALS AND METHODS

Materials

[4-^{14}C]cholesterol, [2-^{3}H]DPPC, and 1-palmitoyl-2-oleoyl[1-^{14}C]PC were purchased from New England Nuclear (Boston, MA). Phospholipase D prepared from Savoy cabbage was a gift from Drs. D. R. Voelker and M. K. Storey (Anna Perahia Adatto Clinical Research Center, Denver, CO). Bio-Sil A (100-200 mesh) was from Bio-Rad (Richmond, CA). Unless indicated, all other chemicals and reagents were from BHD (Poole, UK). Concentrations of ^{3}H and ^{14}C radioactivities were verified with a scintillation counter (LS 6000 5C; Beckman, Fullerton, CA) using Beckman ReadySolve HP scintillation fluid. Distilled water purified through a Millipore (Danvers, MA) Milli-Q four-cartridge system was used in all experiments.

Preparation of BLES(chol)

BLES(chol) was obtained from natural bovine pulmonary surfactant, kindly provided by BLES Biochemicals, through chloroform-methanol extraction by the method of Bligh and Dyer (49), as described previously (12). BLES(chol) retains all lipid components of surfactant and surfactant proteins, SP-B and SP-C, but not SP-A or SP-D. BLES(chol) differs from BLES® used clinically, which has the neutral lipids removed (20).

Preparation of [^{14}C]DPPG and [^{14}C]POPG

[^{14}C]DPPG and [^{14}C]POPG were prepared from the corresponding [^{14}C]PCs through transphosphorylation catalyzed by phospholipase D using a modification of the method of Comfurius and Zwaal (50). Briefly, 100 μg [^{14}C]PC in 200 μl diethylether was mixed with 100 μg phospholipase D in 100 μl buffer of 200 mM sodium acetate-$CaCl_2$, pH 5.6, and 100 μl 50% glycerol in the same buffer. The mixture was shaken vigorously with a Multi-Mixer (Lab Line Instruments, Inc., Melrose Park, IL) at room temperature for about 1 h until the yield of [^{14}C]PG was at least 80%, as detected by TLC using a solvent system (chloroform-methanol-water-triethylamine; 30:34:8:35, v/v/v/v) described by Touchstone et al. (51). The reaction was stopped with 100 μl of 200 mM EDTA. Diethylether was evaporated and the [^{14}C]phospholipids were extracted with chloroform-methanol following the procedure of Bligh and Dyer (49). The [^{14}C]PGs were purified by silicic acid column chromatography. The column was washed with 10 vol of chloroform. The [^{14}C]phospholipids dissolved in chloroform were applied into the column and eluted with a step-gradient of chloroform-methanol. The [^{14}C]PGs were eluted with chloroform-methanol, 9:1 (v/v). Radiolabeled purity was confirmed by TLC, as above.

Preparation of samples

The desired amount of BLES(chol) was mixed with [^{3}H]DPPC and either [^{14}C]POPC, [^{14}C]DPPG, [^{14}C]POPG, or [^{14}C]cholesterol in chloroform-methanol, 9:1 (v/v). The solvent was evaporated under N_2, and the residue was hydrated with 150 mM NaCl-1.5 mM $CaCl_2$ and 5 mM Hepes, pH 7.4 (1 mg/100μl). The suspension was shaken with a Multi-Mixer at room temperature for about 100 min and incubated at 37°C for 2–3 h. Ten microliter samples were transferred to a counting vial as a sample of the initial dispersion before injection into dish 1 (D1, see the next section). The specific radioactivity of the [^{3}H]DPPC used was 0.5–1.5 μCi/mg lipid, and that of [^{14}C]POPC, 0.25–0.5 μCi/mg; [^{14}C]DPPG, 0.5 μCi/mg; [^{14}C]POPG, 0.5 μCi/mg; and cholesterol, 0.5–1 μCi/mg lipid.

Isolation of monolayers from dispersions

Two Teflon dishes, dish 1 (D1) and dish 2 (D2), containing 150 mM NaCl-1.5 mM $CaCl_2$ and 5 mM Hepes (pH 7.4) in a 37°C water bath, were connected with a 1 × 2.5 cm^2 strip of ashless filter paper that was suspended with a Teflon tape-wrapped wire (**Fig. 1**). A 5 mm-wide platinum plate dipped into D2 served to monitor surface tension. The surface area of D1 was 2, 2.5 or 20 cm^2 and that of D2 was 10, 12.5, or 20 cm^2, providing surface area ratios for D1-D2 of 2:1, 1:1, and 1:10. After 30 min of equilibration, a 37°C preincubated sample of [^{3}H]DPPC and [^{14}C]POPC, [^{14}C]DPPG, [^{14}C]POPG, or [^{14}C]cholesterol-labeled BLES(chol) was injected into D1. The final concentration of BLES(chol) in D1 was 0.15 mg/ml for D1-D2 = 2:1, 0.25 mg/ml for D1-D2 = 1:1, and 0.5 mg/ml for D1-D2 = 1:10. The filter paper was removed within 15 min after the equilibrium surface tension (~24 mN/m) in D2 was reached. All experiments were performed in a temperature-controlled box at 37 ± 0.5°C. The surface monolayer in D2 was transferred with a pipette into a test tube, and lipids were extracted with chloroform-methanol, 1:1 (v/v) (49). The chloroform layer was transferred to a counting vial, and solvent was evaporated under N_2. Twenty microliters of the dispersion from the bulk (D1) were taken and transferred to a counting vial at the end of each experiment. Five microliters of scintillation fluid was added into all vials, and the values of [^{3}H]DPPC-[^{14}C]lipid were obtained from the scintillation counter. Radioactive counting was continued to preset errors of <1%.

L-B films from dispersions of [^{3}H]DPPC- and [^{14}C]POPC-labeled BLES(chol)

L-B films were deposited from adsorbed dispersions of [^{3}H]DPPC- and [^{14}C]POPC-labeled BLES(chol) on 1 × 1 cm^2 microscope glass cover slips at 37°C as described previously (12). Surface tension was maintained at 24 mN/m during deposition. The films were eluted with chloroform-methanol, 1:1 (v/v), into a counting vial. Solvent was evaporated under N_2, and the residues were dissolved in 5 ml scintillation fluid. Values of [^{3}H]DPPC-[^{14}C]POPC were obtained from scintillation counting.

RESULTS

DPPC and POPC in L-B films from BLES(chol) dispersions

Initial experiments investigated the transfer of radiolabeled PCs from the bulk phase to the air-water interface during adsorption. L-B films were deposited from dispersions of [^{3}H]DPPC- and [^{14}C]POPC-labeled BLES(chol). **Table 1** reveals that the eluted L-B films contained DPPC to POPC ratios similar to those in the initial dispersions and in samples recovered from the bulk phase. In previous studies, we have observed that the L-B films deposited in this manner contained more radioactivity than could arise from a single monolayer (12). In addition, X-ray autoradiography revealed the presence of highly intense radioactive areas, suggesting lipid aggregates. These observations indicated that L-B films prepared from adsorbed BLES(chol) films include both the surface monolayer and associated material. The present data indicate that the relative concentrations of DPPC and POPC in the adsorbed films are same as in the bulk phase and are representative of the original injected dispersions.

DPPC-POPC ratios in surface films spread from BLES(chol)

Table 2 displays [^{3}H]DPPC-[^{14}C]POPC ratios in surface films recovered from spreading dish D2 that arise after injection of [^{3}H]DPPC-[^{14}C]POPC-labeled BLES dispersions into adsorbing dish D1. With surface areas D1 = D2 (Table 2), the ^{3}H-^{14}C ratios of the recovered surfactant were within a percentage point of that of the original dispersion and of BLES(chol) recovered from the bulk phase of D1 after equilibrium surface tension had been achieved. This is within the counting error of the procedures used. These results show that POPC is adsorbed into the surface film along with DPPC. It is also apparent that the relative concentrations of DPPC and POPC in the spread films transferred to D2 are similar to those of the BLES(chol) samples remaining in the adsorption dish D1 after adsorption.

The data in Table 2 were obtained from films in spreading dish D2 that had 10 times the surface area of D1. Similar to the condition D1 = D2, the [^{3}H]DPPC-[^{14}C]POPC ratios were comparable to the initial dispersion and BLES(chol) recovered from the bulk phase. These results show that the relative amounts of DPPC and POPC in films in spreading dish D2 remain unchanged where 10

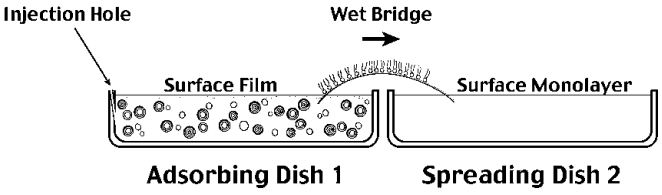

Fig. 1. Diagrammatical representation of the wet-bridge lipid transfer procedure. The surfactant sample is injected through the injection hole into the subphase of adsorbing dish D1. The injected surfactant adsorbs to equilibrium surface tension (~24 mN/m) as a monolayer and its associated reservoir. The film spreads across the filter paper-supported wet bridge to the surface spreading dish D2, where it becomes a monolayer. Both dipalmitoyl-phosphatidylcholine (DPPC) (dark) and non-DPPC (white) lipids are transferred. Spreading continues until equilibrium surface tension is attained in D2.

TABLE 1. [^{3}H]DPPC-[^{14}C]POPC isotopic ratios in L-B films deposited from [^{3}H]DPPC and [^{14}C]POPC labeled dispersions of BLES(chol)

Initial Dispersions[a]	L-B Films[b]	% of Initial Dispersion	Bulk Phase[c]	% of Initial Dispersion
1.819	1.826	(100.4)	1.821	(100.1)
2.234	2.258	(101.1)	2.258	(101.1)
2.712	2.760	(101.8)	2.669	(98.4)
		101.1 ± 0.40 (n = 3)		99.9 ± 0.78 (n = 3)

[a] "Initial Dispersions" indicates samples hydrated and incubated at 37°C prior to injection into adsorbing dish D1. Three individual samples were prepared and each was tested once.

[b] L-B films were eluted with chloroform-methanol, 1:1 (v/v), and radioactivity determined by scintillation counting.

[c] Bulk phase samples were taken from the subphase of the adsorbing dish after deposition of the L-B films.

TABLE 2. Comparison of [^{3}H]DPPC-[^{14}C]POPC ratios in monolayers in D2 spread from adsorbed films of [^{3}H]DPPC and [^{14}C]POPC labeled BLES(chol) dispersion injected into D1 and BLES (chol) remaining in the bulk phase of D1

Surface Areas of D1-D2 = 1:1				
Initial Dispersion[a]	Monolayer[b]	% of Initial Dispersion	Bulk Phase[c]	% of Initial Dispersion
0.693	0.700	(101.1)	0.687	(99.5)
1.790	1.755	(98.1)	1.771	(98.9)
2.547	2.574	(101.1)	2.534	(99.5)
		100.1 [d] ± 1.01 (n = 3)		99.3[d] ± 0.18 (n = 3)
Surface Areas of D1-D2 = 1:10				
Initial Dispersion[a]	Monolayer[b]	% of Initial Dispersion	Bulk Phase[c]	% of Initial Dispersion
2.250	2.259	(100.4)	2.233	(99.3)
2.702	2.712	(100.4)	2.688	(99.5)
3.087	3.112	(100.8)	3.077	(99.6)
		100.6 [d] ± 0.13 (n = 3)		99.4[d] ± 0.09 (n = 9)

[a] Initial dispersions are samples hydrated and incubated at 37°C before injecting into adsorbing dish 1 (D1). Three separate samples were prepared and each was tested once.
[b] Monolayers (D2) spread from adsorbed films (D1) were suctioned and extracted with chloroform-methanol, 1:1 (v/v).
[c] Bulk samples were taken from the subphase of D1 after removing the paper bridges between D1 and D2.
[d] Values are mean ± SE.

times as much surfactant material is transferred across the wet bridge.

DPPC-DPPG ratios in surface films spread from BLES(chol)

DPPG is present at low levels in pulmonary surfactant, and this anionic lipid can achieve low surface tensions near 0 mN/m during compression (52). [^{3}H]DPPC, prepared enzymatically using PLD, was used to examine the effect of phospholipid headgroup on adsorption into surface films. Similar to the results on DPPC and POPC (Table 2), identical ^{3}H-^{14}C ratios were observed in films recovered from spreading dish D2 as in the original injected dispersions and in BLES(chol) recovered from the bulk phase of adsorbing dish D1 (**Table 3**). The radioactive ratio remained constant, regardless of whether the D1-D2 surface ratio was 1:1 or 1:10. These results confirm that DPPG is adsorbed into surface films along with DPPC and that the relative concentrations of these two gel phase phospholipids transferred to spreading dish D2 corresponded to those in the original BLES(chol) samples injected into D1.

DPPC-POPG ratios in surface films spread from BLES(chol)

The effect of phospholipid headgroup specificity was further examined using [^{14}C]POPG prepared enzymatically. The data presented in **Table 4** reveal [^{3}H]DPPC-[^{14}C]POPG ratios in films from spreading dish D2 were identical to the isotopic ratios in the original dispersions and in BLES(chol) recovered from the subphase of adsorbing dish D1. The radioactive ratio remained constant regardless of whether the transferred films were spread from adsorbing surfaces two times, equal, or one-tenth the surface area of spreading dish D2. Thus, the relative concentrations of DPPC and POPG in the films spread to D2 remained unaltered over a 20-fold range of adsorbing

TABLE 3. [^{3}H]DPPC-[^{14}C]DPPG ratios in monolayers, bulk phases, and initial dispersions

Surface Areas of D1-D2 = 1:1				
Initial Dispersion	Monolayer	% of Initial Dispersion	Bulk Phase	% of Initial Dispersion
1.125	1.157	(102.8)	1.117	(99.3)
1.627	1.589	(97.7)	1.614	(99.2)
1.842	1.860	(101.0)	1.845	(99.9)
		100.5 ± 1.51 (n = 3)		99.5 ± 0.20 (n = 3)
Surface Areas of D1-D2 = 1:10				
Initial Dispersion	Monolayer	% of Initial Dispersion	Bulk Phase	% of Initial Dispersion
1.453	1.467	(101.0)	1.455	(100.1)
1.549	1.574	(101.6)	1.540	(99.4)
1.650	1.684	(102.0)	1.641	(99.5)
		101.5 ± 0.31 (n = 3)		99.7 ± 0.23 (n = 3)

Experiments were performed as in Table 2.

TABLE 4. [^{3}H]DPPC-[^{14}C]POPG ratios in monolayers, bulk phases, and initial dispersions

Surface Areas of D1-D2 = 2:1				
Initial Dispersion	Monolayer	% of Initial Dispersion	Bulk Phase	% of Initial Dispersion
1.893	1.927	(101.8)	1.919	(101.4)
2.331	2.356	(101.1)	2.350	(100.8)
3.585	3.549	(99.0)	3.567	(99.6)
		100.6 ± 0.84 (n = 3)		100.6 ± 0.54 (n = 3)
Surface Areas of D1-D2 = 1:1				
Initial Dispersion	Monolayer	% of Initial Dispersion	Bulk Phase	% of Initial Dispersion
1.629	1.630	(100.1)	1.644	(100.9)
1.748	1.764	(100.9)	1.750	(99.9)
1.843	1.855	(100.7)	1.828	(99.0)
		100.5 ± 0.26 (n = 3)		99.9 ± 0.55 (n = 3)
Surface Areas of D1-D2 = 1:10				
Initial Dispersion	Monolayer	% of Initial Dispersion	Bulk Phase	% of Initial Dispersion
1.527	1.544	(101.1)	1.498	(98.8)
2.028	2.072	(102.2)	2.027	(100.0)
3.171	3.125	(101.5)	3.164	(99.8)
		101.6 ± 0.31 (n = 3)		99.5 ± 0.35 (n = 3)

Experiments were performed as in Table 2.

to spreading surface areas and a 3.3-fold variation in surfactant bulk concentration.

DPPC-cholesterol ratios in surface films spread from BLES(chol)

Although its role remains ambiguous, cholesterol is present in small amounts in all mammalian surfactants examined and in surfactants from birds and reptiles (7, 53). We previously presented evidence indicating cholesterol was adsorbed along with DPPC into the initial surface monolayers formed from BLES(chol) (13). The present results shown in **Table 5** are consistent in that they reveal [^{14}C]cholesterol is adsorbed into the surface films with [^{3}H]DPPC. The ^{3}H-^{14}C ratios in films transferred to spreading dish D2 were identical to those in the injected sample and to those of BLES(chol) recovered from the subphase of D1. Similar results were obtained whether the films were transferred from surfaces with equal or one-tenth the area of the spreading dish D2. These results confirm cholesterol is adsorbed along with DPPC into surface films and indicate neutral lipids behave similarly to DPPC and to non-DPPC phospholipids.

DISCUSSION

All known pulmonary surfactants are rich in DPPC, and DPPC is the only major component of surfactant that can attain low surface tensions during monolayer dynamic lateral compression under normal conditions (3–7, 33). The generally accepted "classical" model for surfactant function proposes that the lung is stabilized by an interfacial monolayer that is highly enriched in DPPC (35, 39–41). The potential mechanism suggested for DPPC enrichment was that adsorbed surfactant monolayers reflect the bulk composition, but during dynamic lateral compres-

TABLE 5. [^{3}H]DPPC-[^{14}C]cholesterol ratios in monolayers, bulk phases, and initial dispersions

Surface Areas of D1-D2 = 1:1				
Initial Dispersion	Monolayer	% of Initial Dispersion	Bulk Phase	% of Initial Dispersion
0.623	0.636	(102.0)	0.624	(100.2)
1.276	1.276	(100.0)	1.273	(99.8)
1.905	1.923	(100.9)	1.892	(99.3)
		101.0 ± 0.58 (n =3)		99.8 ± 0.24 (n = 3)
Surface Areas of D1-D2 = 1:10				
Initial Dispersion	Monolayer	% of Initial Dispersion	Bulk Phase	% of Initial Dispersion
1.052	1.047	(99.9)	1.053	(100.0)
1.926	1.955	(101.5)	1.917	(99.5)
2.285	2.300	(100.7)	2.282	(99.9)
		100.70 ± 0.45 (n = 3)		99.8 ± 0.13 (n = 3)

Experiments were performed as in Table 2.

sion, the more fluid non-DPPC phospholipid components are squeezed out of the surface monolayer. The resulting DPPC enrichment is considered necessary in order for the monolayer to achieve the low surface tensions required to stabilize the lung. Recently, a number of physicochemical studies have demonstrated that low surface tension can be achieved with smaller surface area reductions than would be predicted by the squeeze-out mechanism (43–46). It has consequently been proposed that sorting or refining of surfactant lipids occurs during adsorption so as to generate a monolayer already enriched in DPPC (3–7, 37, 38).

Recent results from Hall's laboratory have challenged the classical theory whereby alveolar stability requires a DPPC-enriched monolayer. Piknova et al. have used fluorescence-labeled and Brewster angle microscopy to show that the phospholipid fraction from calf lung surfactant extracts can attain very low surface tensions without significant monolayer enrichment in DPPC (54). In addition, Crane et al. have observed that when spread monolayers of dimyristoylphosphatidylcholine or POPC were compressed at rates comparable to those in the lung alveolar surface, surface tensions ranging from below equilibrium (~24 mN/m) to near 0 could be achieved (55–57). Although thermodynamically metastable, such films remain at surface tensions below equilibrium for prolonged periods. These workers concluded that "kinetically-trapped" transformed surfactant monolayers could persist at very high surface pressures (low surface tensions) without surface refinement of their components. In agreement with these conclusions, we have observed that cholesterol, a known fluidizing agent, persists in autoradiographs of L-B films deposited from spread monolayers of DPPC/cholesterol/SP-A at surface tensions of ~1 mN/m. However, whether the cholesterol is a constituent of the surface monolayer or is associated with the reservoir was not clear from those studies (58).

We have previously studied film formation by injecting ^{14}C-radiolabeled BLES near the bottom of Teflon dishes and measuring surface tension with a Wilhelmy plate. Surface radioactivity was monitored with a scintillation probe positioned just over the water surface (12, 58). Since ^{14}C emissions have a range in water of ~0.25 mm, the surface radioactivity probe measures radioactivity in the surface region, not just the surface film. In these experiments, the surfactant lacked sufficient radioactivity for detection of a single monolayer. BLES(chol) at 0.1, 0.2, and 0.3 mg/ml (final concentrations) adsorbed rapidly and attained the equilibrium surface tension of 24 mN/m in 30, 5, and 2 min, respectively. Surface radioactivity from [^{14}C]DPPC or [^{14}C]cholesterol-labeled BLES increased much more slowly. Further, although, as expected, the final levels of surface radioactivity varied, in all three cases surface radioactivity equilibrated at ~120 min. The differences in time course and the effect of concentration were consistent with a mechanism in which surface vesicles interacting with the interface release phospholipids to form a film that spreads rapidly to cover the available surface. As a result, surface equilibration occurs long before equilibration of surfactant concentrations in the bulk phase. It has previously been shown that dry surfactant particles and dry phospholipid mixtures sprinkled on an aqueous surface spread rapidly as they equilibrate (59). The above interpretation is consistent with studies with Curosurf® that showed this therapeutic surfactant adsorbs in rapid bursts (60). Subsequent studies using the wet-bridge system revealed the time courses for surface tension reduction in adsorbing dish D1 were similar to those in the film-receiving dish, D2 (13). As in the case of the earlier studies where only adsorbing dishes were employed, the time courses were highly concentration dependent. These observations are consistent with the suggestion that formation of the surfactant film in dish D1 provides the driving force for spreading of the surfactant film across the wet bridge onto the surface of dish D2.

In the present studies, we have investigated surfactant film composition by employing a paper-supported wet bridge to transfer surfactant films from the surface of an adsorbing dish, D1, to the surface of a spreading dish, D2. Because the absolute amounts of lipid transferred to the spreading dish are very small, even at the equilibrium surface tension of 24 mN/m, we used radioactive [^{3}H]DPPC in combination with other ^{14}C lipids to monitor the relative concentrations of these surfactant components in the dispersions and surface films. Using this approach, we observed the [^{3}H]DPPC-[^{14}C]POPC ratio of L-B films deposited on glass was identical to that of the original BLES(chol) dispersions. Previous studies have shown that such L-B films contain both the surface monolayer and associated material (12, 13, 61). However, such L-B films do not sample surfactant material injected under prespread films. Whether all of the surfactant material associated with surfactant monolayers can act as a functional reservoir is not known. Nevertheless, this experiment shows that there is no substantial selectivity for DPPC incorporation into entire surface films during adsorption.

The above approach was extended to examine the relative compositions of surface films transferred from the adsorbing dish, D1, to the spreading dish, D2, by a thin filter paper-supported wet bridge. Previous experiments have shown that a rapid increase in the surface area of D2 (after removal of the wet paper bridge) generates immediate, proportionate increases in surface tension. These and other observations by our group and others are consistent with the film in D2 being a monolayer (13, 47, 48). This indicates that, as the film is transferred from a region of high surface pressure in D1 to a lower surface pressure in D2, it forms a single monolayer. Surfactant material from the surface-associated reservoir in D1 continues to spread into the surface monolayer and serves to drive lipid transfer across the wet bridge until equilibrium surface tension is attained.

The wet-bridge approach was used to study relative [^{3}H]DPPC-[^{14}C]POPC transfer of BLES(chol) injected under the surface in adsorbing dish D1 to spread equilibrium films on the surface of D2. It was observed that the ^{3}H-^{14}C ratio of the transferred film was identical to the isotopic ratio of the injected BLES(chol) dispersions and of BLES(chol) recovered from the subphase of D1. Taken to-

gether with the L-B studies discussed above, this would indicate that similar DPPC-POPC relative concentrations were maintained during formation of the surface film and its subsequent transfer across the wet-paper bridge. Furthermore, this relationship was maintained over a wide range of BLES(chol) concentrations of 0.25–0.5 mg/ml, with 0.15 mg/ml used in some preliminary studies. The same relationship was maintained for spread films at equilibrium in D2, with relative surface areas for D1 and D2 varying from 1:1 to 1:10. These results demonstrate a lack of DPPC selectivity over POPC in surface film formation and transfer. Identical results were obtained in studies comparing relative ^{3}H-^{14}C levels with [^{3}H]DPPC and [^{14}C]DPPG, [^{14}C]POPG, or [^{14}C]cholesterol, showing that these non-DPPC lipids acted uniformly (relative to DPPC) during adsorption and transfer. The following considerations on the surface origin (i.e., monolayer or reservoir) of the material transferred to the spreading dish, D2, should therefore apply in all cases.

It is anticipated that under conditions in which the surface area of D1 = D2, some of the monolayer spread to D2 will be derived from the original adsorbed monolayer, and some from the monolayer-associated reservoir in D1. This occurs because film spreading into D2 allows incorporation of reservoir surfactant into the surface monolayers in D1. As a consequence, the monolayer spreading onto dish D2 will contain phospholipid from the original adsorbed equilibrium monolayer and an unknown proportion of phospholipid introduced into the monolayer in D1 from its reservoir. Considerable evidence indicates reservoir formation is highly dependent on surfactant bulk concentration (6, 10, 37). The observation that altering surfactant bulk concentration from 0.15 to 0.5 mg/ml did not affect the DPPC-nonDPPC lipid isotopic ratio in D2 is consistent with identical relative concentrations for these phospholipids in the bulk adsorbed monolayer and the accessible reservoir in D1. The conditions used for these experiments were chosen so that equilibrium surface tension was achieved in the spreading dish D2 ~5 min after injection of the BLES(chol) sample in D1. These conditions were chosen in order to minimize the possibility of surface radioactivity equilibration due to diffusion of radiolabeled phospholipid molecules in the monolayers. Identical results were obtained when the spread monolayers were allowed to remain for 30 min or more after equilibrium surface tension was attained (not shown).

As indicated above, with surface area D1 = D2, the film spreading into D2 would arise partially from the monolayer in D1. With surface area D1-D2 = 2:1, the proportion of spread monolayer in D1 arising from the original adsorbed monolayer in dish D1 will increase. However, with surfactant area D1-D2 = 1:10, at least 90% of the spread monolayer in D2 would arise from reservoir material in D1. This transferred reservoir material would correspond to at least 4.5 phospholipid bilayers associated with the adsorbed monolayer in D1. Although the 1:20 ratio variation in surface area was investigated only with [^{3}H]DPPC-[^{14}C]POPG, the overall results with all lipid combinations were very consistent. The similarity of the DPPC-nonDPPC lipid ratio with the injected radiolabeled BLES samples under all of the experimental conditions provides compelling evidence against relative increases in DPPC content of the original adsorbed monolayer.

The above arguments depend on relative homogeneity of the surface film. It is recognized that spread and adsorbed films containing gel phase phospholipids can spontaneously form liquid condensed domains enriched in DPPC (54, 62–64) [as reviewed in ref. (19)]. Such domains are quite small and appear rather evenly distributed over the surface of the film and should not influence the relative measurements of DPPC-nonDPPC lipid ratios reported here. Any selective transfer of domains would be detected as an increase in ^{3}H-^{14}C ratio, while discrimination against domain transfer would appear as a decrease in this ratio. The lack of such alterations would argue against any selectivity in lipid transfer related to lipid domains.

In summary, these studies have examined the relationship between the lipid composition of adsorbed monolayers and their associated reservoirs and compared them to the lipid composition of the radiolabeled lipid extract surfactant injected into the bulk phase. The relative concentrations of [^{3}H]DPPC and ^{14}C-labeled POPC, DPPG, and POPG were examined to investigate the effect of polar headgroup and acyl-chain specificity on putative DPPC selective adsorption. [^{14}C]cholesterol was used to determine the effect of lipid class. The reported investigations studied the effect of varying bulk surfactant concentrations of 2- to 3.3-fold. Bulk surfactant concentration has a major impact on surface activity and reservoir formation. These investigations also studied the effect of varying the relative surface areas of adsorbing D1 to the spreading D2 dishes by 10- and, in the case of POPG, 20-fold. The studies show a uniform lack of lipid selectivity in the formation of surface films during adsorption. More importantly, they failed to detect any evidence for DPPC enrichment during generation of adsorbed monolayers. These results are counter to the suggestion that lipid sorting occurs during surfactant monolayer formation. They are consistent with recent evidence arguing against the classical model, which has proposed that the ability of pulmonary surfactant to attain surface tensions near 0 mN/m is dependent on monolayers highly enriched in DPPC.

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