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ARTIFICIAL PULMONARY SURFACTANT

POTENTIAL ROLE FOR HEXAGONAL H_{II} PHASE IN THE FORMATION OF A SURFACE-ACTIVE MONOLAYER

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Natural surfactant possesses the ability to rapidly reduce the surface tension of a bubble pulsating at 20 cycles per min at 37°C to less than 30 dyn/cm at maximum radius and to less than 1 dyn/cm at minimum radius. The preparation of two artificial surfactant systems, containing either dipalmitoylphosphatidylcholine (DPPC) and phosphatidylethanolamine (PE) (5:5), or DPPC plus PE and phosphatidylglycerol (5:4:1 or 6:3:1), is described. Formation of artificial surfactants which mimic the essential properties of natural surfactant was correlated with the appearance of particles of aggregated lipids. The effects of lipid composition, calcium ion concentration, pH, temperature and mechanical agitation were determined. It is proposed that these artificial surfactant systems may produce a surface-active monolayer through the involvement of nonbilayer structures with properties similar to hexagonal H_{II} phase.

Introduction

The mammalian lung is stabilized by an extraordinary material, the pulmonary surfactant, which reduces the surface tension at the air-liquid interface of the alveoli [1–3]. Studies using isolated lungs indicate that in the presence of surfactant, the surface tension in normally expanded lungs approximates 30 dyn/cm, while during expiration this value approaches 0 dyn/cm [4–6]. Presence of pulmonary surfactant is particularly critical at birth when the newborn infant must clear its lungs of pulmonary fluid and establish regular breathing. Absence of sufficient surfactant stores to maintain a low surface tension during the neonatal period appears to be the major factor associated with the development of the neonatal respiratory distress syndrome, the principal cause of perinatal mortality and morbidity in developed countries [7,8]. Treatment of infants suffering from advanced neonatal respiratory distress syndrome with surfactant preprations derived from bovine surfactant lipid extracts or human amniotic fluid [9–11] results in a marked improvement in lung expansion and gaseous exchange.

It is generally acknowledged that the ability of pulmonary surfactant to reduce the surface tension of an air/liquid interface to near 0 dyn/cm is dependent upon the formation of a monolayer of

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Abbreviations: DPH, 1,6-diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine; I, fluorescence intensity; H_{II}, hexagonal H_{II} phase; Δp, pressure differences across a bubble; P, fluorescence polarization; r, fluorescence polarization anisotropy; R_{max}, radius at maximum bubble radius; R_{min}, minimum bubble radius.

relatively pure dipalmitoylphosphatidylcholine (DPPC) during compression [1–3,12]. At 37°C, hydrated bilayers of DPPC exist in the gel state from which adsorption can occur only very slowly [1–3]. The other lipids present in natural surfactant could serve to fluidize the disaturated phosphatidylcholine. In addition, recent studies have demonstrated that the apoproteins present in pulmonary surfactant facilitate the adsorption of DPPC to the air/liquid interface [13–15]. However, although surfactant approteins can enhance DPPC adsorption, the realization that only DPPC and possibly disaturated phosphatidylglycerol (PG) appear to have a role in surface tension reduction has prompted various workers to attempt to develop artificial mixtures which could function to transfer DPPC to the air/liquid interface at a sufficient rate to maintain normal lung function. The availability of functional synthetic surfactants would have obvious economic advantages. One approach towards the formation of artificial surfactants has been to device methods for maintaining DPPC in a nonbilayer phase using long-chain alcohols [16], inert hydrocarbon oils [17], or by administering 'dry' lipid mixtures in which the DPPC is not yet fully hydrated [18]. Another possibility would be to disperse the DPPC with those lipids which promote the formation of hexagonal H_{II} phase [19]. This latter state [20] consists of elongated cylinders of lipids in inverted micellar form with the fatty acids extending outwards and the polar headgroups binding an inner core or pore of water which extends along the elongated cylinder. Since air is more hydrophobic than water, a cylinder of H_{II} phase interacting with the air/liquid interface could tend to unfold, thereby transferring many lipid molecules to the surface. The present paper describes a series of investigations demonstrating the potential for producing effective artificial surfactants using conditions appropriate for the formation of H_{II} phase.

Materials and Methods

Materials. DPPC and 1-palmitoyl-2-oleoylphosphatidylcholine (1-16:0,2-18:1-PC) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The fluorescent probe, 1,6-diphenylhexatriene (DPH) and ethanolamine hydrochloride were obtained from Alrich, Milwaukee, WI, U.S.A. Other chemicals, reagents and solvents were purchased from Fisher Scientific Ltd., Toronto, Ontario, Canada.

1-16:0,2-18:1-PE and 1-16:0,2-18:1-PG were prepared from the corresponding PC by transphorphorylation catalysed by phospholipase D according to the method of Comfurius and Zwaal [21]. Phospholipase D was extracted from Savoy cabbage, purified by heat treatment and acetone precipitation following the procedure of Davidson and Long [22].

Bovine pulmonary surfactant was obtained through lavaging lungs from freshly slaughtered cattle followed by successive differential centrifugation [23].

Preparation or artificial surfactants and methods for dispersion and sonication of lipid mixtures. Thepure phospholipids, dissolved in chloroform, were mixed in the desired proportions and dried under N_2. Suspensions were prepared by adding the suspending medium (at a pH of 6.5–7.0, unless specified otherwise) and a few glass beads (3 mm diameter). The mixtures were shaken vigorously with a wrist-action shaker at room temperature for 1 h, unless specified. The dispersions were incubated at 37°C for 1–2 h before the ability to reduce surface tension was monitored. Where specified, the dispersions were sonicated in a bath sonicator using a B-12 Branson ultrasonic cleaner.

Surface tension measurements. The ability of the dispersion to reduce surface tension were assayed with a pulsating bubble surfactometer (Surfactometer International, Toronto, Ontario, Canada) as described by Enhorning [24]. Samples are loaded into the sample chamber which has a small capillary open to the atmosphere (Fig. 1). An air/liquid interface is created by withdrawing sufficient fluid to produce a bubble with a radius of 0.55 mm. This bubble is pulsated at 20 cycles per min between this maximum bubble size of 0.55 mm (R_{max}) and a minimum of 0.40 mm (R_{min}). The pressure difference (Δp) across the bubble is continuously monitored by a pressure transducer. The surface tension (σ) at any point can be calculated from the Laplace equation:

$$\Delta p = 2\sigma/R \qquad (1)$$

where R refers to the radius of the bubble. Surface

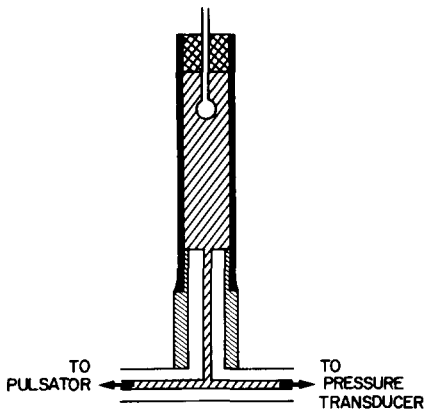

Fig. 1. Diagrammatic representation of the test chamber of the pulsating bubble surfactometer used to assay surface tensions as indicated in Methods.

tension at R_{max} and R_{min} after 60 s pulsation are usually expressed in tables and figures.

Fluorescence polarization determinations. The transition temperatures of individual phospholipids and of mixed dispersions were examined by fluorescence polarization using DPH as the probe [25]. The phospholipids were labelled by the addition of 4 μl of DPH (1 mM in tetrahydrofuran) to 4 ml of dispersion (0.1 mM phospholipid, pH 7.0) while vortexing. The labelled mixtures were sonicated for 30 min at 45 °C with a Branson B-12 sonicator. Fluorescent polarization was measured with a Perkin-Elmer MPF-4 spectrofluorometer. The degree of fluorescence polarization:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (2)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities oriented parallel and perpendicular, respectively, to the direction of polarization excitation light. Fluorescence anisotropy, r, was calculated from:

$$r = 2P/(3 - P) \quad (3)$$

Results and Discussion

Representative pressure tracings obtained with 1% (w/v) phospholipid dispersions of natural surfactant and for artificial surfactants composed of DPPC/PE (5 : 5) and DPPC/PE/PG (5 : 4 : 1) are illustrated in Fig. 2. In each case, the surface tension at R_{max} rapidly fell to 25–30 dyn/cm within a few pulsations after the creation of the bubble. This surface tension corresponds to the equilibrium surface tension for monolayers of DPPC in the absence of external compression [2,3]. By the 10th pulsation (30 s), the surface tensions at minimum bubble radii (R_{min}) approached 0 dyn/cm. In order to display more detail in the pressure tracings, the recorder speeds were accelerated for a few pulsations at the 15th pulsation for natural surfactant and for the DPPC/PE/PG mixture and at the 20th pulsation for the DPPC/PE mixture. With all three samples the pressure tracings maintained a broad flattened plateau with P near 0 cm water. This indices that relatively pure monolayers of DPPC were achieved during compression [12,18]. It was observed that when the surface tensions approached 0 dyn/cm at R_{min} the bubbles flattened somewhat or flopped to one side of the central capillary because it was not possible to maintain their spherical shape at low surface tensions. This phenomenon, which was observed with all three samples, constitutes a separate indicator of extremely low surface tensions [6,17].

The pressure tracing for natural surfactant was reasonably symmetrical and approached a sinusoidal curve. The major inconsistency, which arises during the expansion of the bubble, may be due to the insertion of lipids other than DPPC or even the presence of lipid-depleted patches on the bubble surface. This lack of symmetry represents hysteresis between the compression and expansion phases (see Ref. 24 for a fuller explanation). The pressure tracings for the artificial preparations exhibited a more symmetrical tracings for the artificial preparations exhibited a more symmetrical but a less uniform pattern. This may reflect a lower relative proportion of DPPC on the bubble surface. It is apparent that thes artificial mixtures can mimic the ability of natural surfactant to lower the surface tension of the pulsating bubble to 25–30 dyn/cm at R_{max} and near 0 dyn/cm at R_{min} within 60 s pulsation at 37 °C. These essential characteristics will henceforth be referred to as 'surfactant activity'.

Effect of lipid composition and the presence of Ca^{2+} on surfactant activity

Preliminary studies demonstrated that phos-

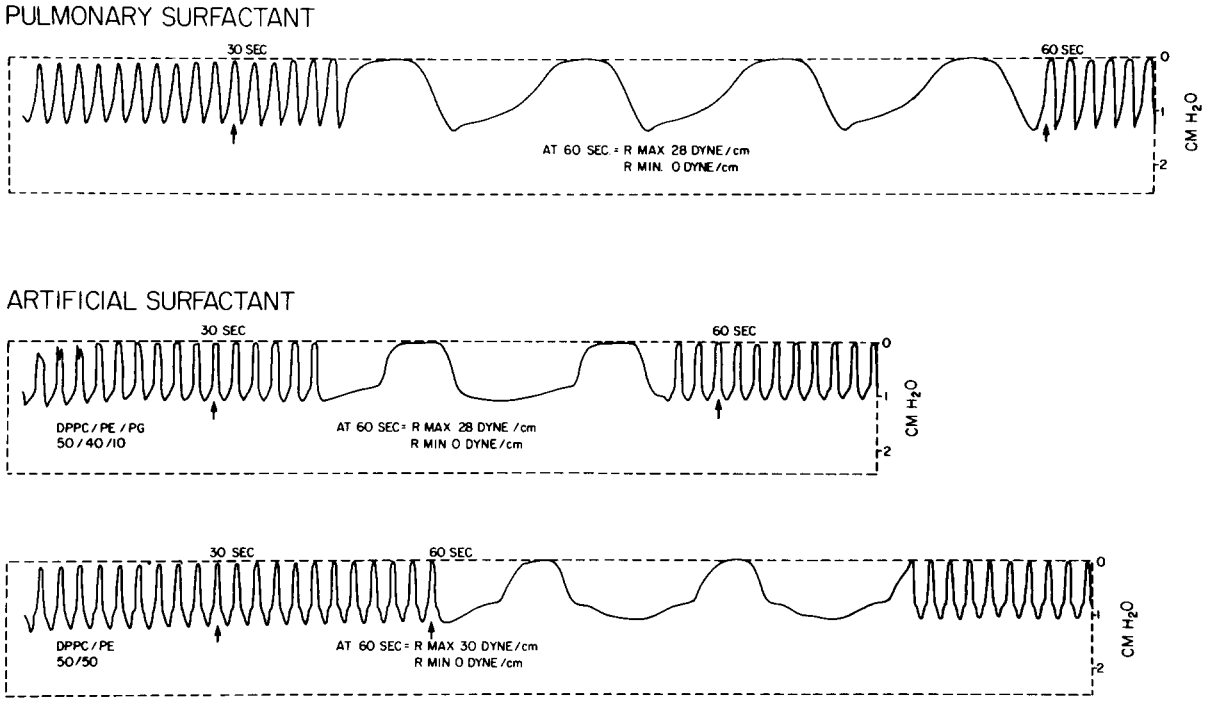

Fig. 2. Representative pressure tracings obtained with the pulsating bubble surfactometer. Bovine pulmonary surfactant in 0.9% NaCl, 1 mM $CaCl_2$ and artificial surfactant DPPC/PE/PG (5:4:1) in 0.9% NaCl, 5 mM $CaCl_2$ and DPPC/PE (5:5) in 0.9% NaCl, 1 mM $CaCl_2$ were pulsated at 37 °C at a concentration of 1% (w/v). Each pressure peak represents one pulsation of 3 sec duration. In order to provide further detail, the chart speeds were increased after approximately 20 pulsations. Surface tensions at maximum radius (R_{max}) and minimum radius (R_{min}) were calculated using the Laplace equation as indicated in the text.

pholipid dispersions possessing the essential characteristics associated with natural surfactant could be formulated from DPPC plus PE from bacterial sources or from egg PE. The ability to consistently produce suspensions with good surfactant activity represents years of investigation involving many thousands of samples. The recognition of considerable batch-to-batch variation with lipids derived from natural sources prompted us to limit the present studies to synthetic lipids with defined acyl-composition. Table I summarizes the effects of varying the lipid composition of the dispersion and the effect of adding increasing amounts of Ca^{2+} on the surfactant activity. As anticipated, dispersions of pure DPPC were relatively ineffective in reducing the surface tension of the pulsating bubble. At R_{max} surface tensions of approx. 50 dyn/cm were observed indicating that insufficient DPPC had absorbed to produce a complete monolayer. The surface tensions at R_{min} were improved by the presence of Ca^{2+} but even at 20 mM, the surface tension at R_{min} remained at 16 dyn/cm. Similar characteristics have been observed with DPPC/PG mixtures [23].

Dispersions of pure 1-16:0,2-18:1-PE produced intermediate surface tension reducing properties. The surface tensions at R_{max} were slightly lower than observed with natural surfactant. However, only a slight decline was produced during compression to R_{min}. This latter effect is presumably related to the inability of the fluid chains of 1-16:0,2-18:1-PE to compress to a sufficiently small mean molecular area to permit the relatively small polar headgroup to bind a large proportion of the free water at the air/liquid interface [3]. The cone shape of PE molecules [20] further mitigates against the production of a tight, compact monolayer. Addition of Ca^{2+} to the suspending medium had no effect on the surfactant properties of pure PE.

Dispersions of DPPC/PE containing 40–70%

TABLE I

EFFECT OF LIPID COMPOSITION AND Ca^{2+} CONCENTRATION ON SURFACE TENSION REDUCTION

Samples (1% (w/v) in a medium comprising 0.9% NaCl and the concentrations of $CaCl_2$ shown) were shaken at room temperature (24 °C) for 1 h and incubated at 37 °C for 1–2 h before assaying with the pulsating bubble apparatus. Surface tension at maximum radius (R_{max}) and minimum radius (R_{min}) after 1 min pulsation at 37 °C are presented as means ± S.E. ($n = 5$).

Composition (% total)			Parameter	Surface tension (dyn/cm)					
				$[CaCl_2]$: 0	1 mM	2.5 mM	5 mM	10 mM	20 mM
DPPC	PE	PG							
100			R_{max}	52 ±3.1	49 ±3.3	48 ±2.7	48 ±2.5	49 ±3.5	48 ±3.7
			R_{min}	30 ±2.0	20 ±1.6	20 ±2.0	20 ±1.5	20 ±2.5	16 ±2.2
	100		R_{max}	22 ±0.4	23 ±0.5	22 ±0.6	22 0.2	22 ±0.4	22 ±0.5
			R_{min}	16 ±0.4	16 ±0.6	15 ±0.7	15 ±0.6	17 ±0.5	16 ±0.5
40	60		R_{max}	28 ±1.0	27 ±0.9	27 ±1.0	27 ±1.0	27 ±1.2	28 ±0.8
			R_{min}	4.0±1.0	4.0±0.6	4.0±0.8	3.0±1.0	4.0±0.5	4.0±0.6
50	50		R_{max}	29 ±1.0	28 ±0.8	29 ±0.8	29 ±1.0	20 ±0.6	28 ±1.0
			R_{min}	0.6±0.5	0.0±0.0	0.0±0.0	0.0±0.0	0.8±0.4	0.6±0.3
60	40		R_{max}	35 ±1.0	36 ±0.8	34 ±1.0	34 ±0.9	34 ±0.7	35 ±0.9
			R_{min}	6.0±0.5	4.0±1.0	5.0±0.7	5.0±0.9	6.0±0.8	6.0±0.6
70	30		R_{max}	38 ±1.5	36 ±1.8	38 ±0.9	38 ±1.0	38 ±1.2	37 ±1.5
			R_{min}	4.0±0.8	6.0±0.5	5.0±1.0	4.0±1.0	5.0±1.2	5.0±0.9
50	40	10	R_{max}	48 ±3.0	40 ±2.5	35 ±1.0	29 ±0.6	29 ±1.0	29 ±0.5
			R_{min}	15 ±1.0	15 ±0.9	8.0±1.0	0.0±0.0	0.0±0.0	0.0±0.0
60	30	10	R_{max}	48 ±2.8	40 ±2.0	36 ±1.0	30 ±1.0	30 ±0.5	30 ±0.7
			R_{min}	14 ±1.5	14 ±1.2	10 ±0.7	0.0±0.0	0.0±0.0	0.0±0.0

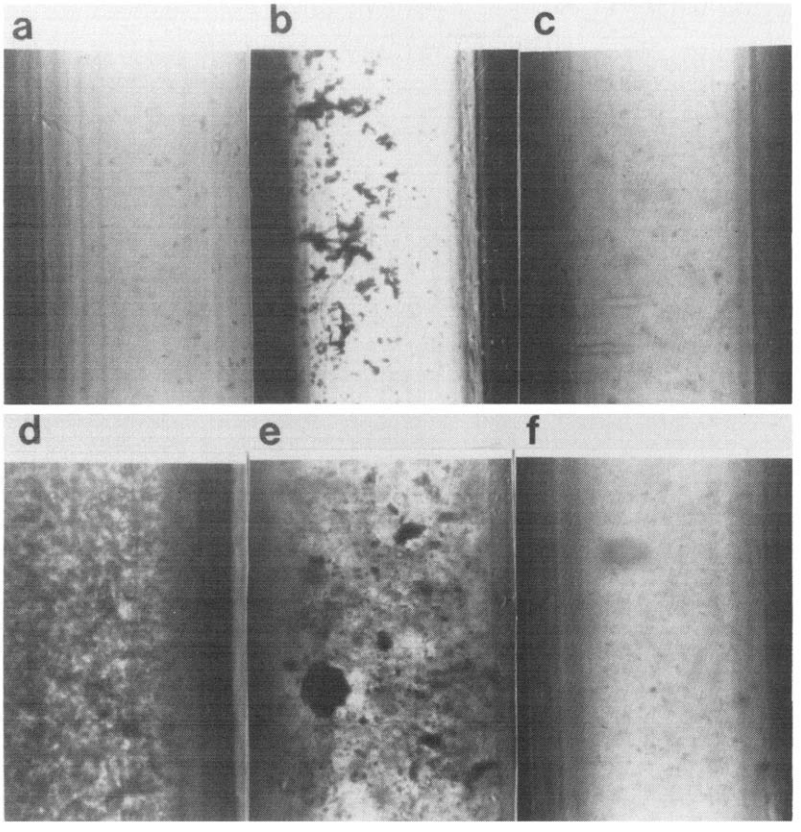

Fig. 3. Micrographs (40×) of samples of bovine pulmonary surfactant and artificial surfactant in the sample chambers of the pulsating bubble surfactometer showing the appearance of aggregated lipidic particles: (a) pulmonary surfactant (1%, w/v) dispersed in 0.9% NaCl, 1 mM $CaCl_2$ and incubated for 1 h at 37 °C; (b) 1-16:0,2-18:1-PE (1%, w/v) dispersed in 0.9% NaCl and incubated at 37 °C for 1 h; (c) DPPC/PE/PG (5:4:1) (1%, w/v) dispersed in 0.9% NaCl, 5 mM $CaCl_2$ and shaken at 24 °C for 1 h; (d) sample from (c) after incubation at 37 °C for 1 h; (e) sample from (d) pulsated at 37 °C for 3 min; (f) sample from (e) bath-sonicated at 24 °C for 10 min and then incubated at 37 °C for 1 h.

disaturated phosphatidylcholine all exhibited good surfactant activity. Optimal surface tension reduction was observed with equal amounts of each lipid. With these preparations, addition of Ca^{2+} up to 20 mM had no effect, presumably because of the low affinity of this divalent cation for neutral phospholipids in the neutral pH range [26,27]. Increasing the DPPC content above 50% resulted in a small but distinct increase in the surface tension at R_{max}.

When 1-16:0,2-18:1-PG was included, rather poor surfactant activities were observed with suspensions in saline. Addition of 5 to 20 mM Ca^{2+} resulted in surfactant characteristics similar to those observed with the natural product.

Relation between aggregated lipid particles and surfactant activity

Natural surfactant forms smooth uniform dispersions (Fig. 3a). Dispersions of 1-16:0,2-18:1-PE tend to flocculate (Fig. 3b). Dispersions of DPPC/PE between 4 : 6 and 7 : 3 formed uniform suspensions at room temperature but flocculated lipid aggregates were observed upon incubation at 37 °C. The appearance of these aggregates correlated with the development of good surfactant activity. Ca^{2+} up to 20 mM did not affect the formation of lipid aggregates or the expressions of surfactant activity.

When part of the PE was replaced by PG, the phospholipid dispersions remained homogeneous when incubated at 37 °C in saline alone. These dispersions displayed poor surfactant activity (Table I). Addition of Ca^{2+} at 5 mM or greater resulted in the appearance of lipid aggregates during incubation at 37 °C and in a marked enhancement of the ability to reduce the surface tension at R_{min} (Table I). Since both the appearance of lipid aggregates and surfactant activity were optimal with DPPC/PE (5 : 5) and DPPC/PE/PG (5 : 4 : 1 and 6 : 4 : 1) in the presence of 5 mM $CaCl_2$, these lipid mixtures were selected for further studies.

Temperature dependence of the formation of lipidic particles

It has been reported that the bilayer to H_{II} phase transition of unsaturated PE occurs within 15 °C above the high temperature end of the hydrocarbon phase transition [28]. The hydrocarbon phase transition profiles of single and mixed phos-

TABLE II

EFFECT OF INCUBATION AT 37 °C ON SURFACTANT ACTIVITY

Mixed phospholipids in a medium comprising 0.9% NaCl and the concentrations of $CaCl_2$ shown were shaken at 24 °C for 1 h and then assayed immediately (A) at 37 °C as indicated in the Methods. Aliquots were also assayed after incubation at 37 °C for 1 h (B) in the same medium. Results are presented as mean ± S.E. ($n = 5$).

Composition (%)				Parameter	Surface tension (dyn/cm)				
DPPC	PE	PG			[$CaCl_2$]: 0	1 mM	5 mM	10 mM	20 mM
50	50	0	(A)	R_{max}	45 ±3.0	45 ±2.6	43 ±3.1	43 ±2.9	44 ±2.0
				R_{min}	8.0±1.0	8.0±1.2	7.0±1.4	6.0±1.8	7.0±1.0
			(B)	R_{max}	29 ±1.0	28 ±0.8	29 ±1.0	29 ±0.6	28 ±1.0
				R_{min}	0.6±0.5	0.0±0.0	0.0±0.0	0.8±0.4	0.6±0.3
50	40	10	(A)	R_{max}	48 ±2.5	40 ±1.5	38 ±1.6	37 ±1.8	38 ±1.0
				R_{min}	14 ±0.8	15 ±0.6	10 ±1.5	8 ±1.0	4 ±1.2
			(B)	R_{max}	48 ±1.8	40 ±2.0	29 ±0.6	29 ±0.8	29 ±0.9
				R_{min}	15 ±1.0	15 ±0.9	0.0±0.0	0.0±0.0	0.0±0.0
60	30	10	(A)	R_{max}	47 ±2.0	42 ±1.8	37 ±1.0	38 ±1.2	38 ±1.0
				R_{min}	14 ±1.0	15 ±0.8	8 ±0.9	6 ±1.1	5 ±1.3
			(B)	R_{max}	47 ±2.8	40 ±2.0	30 ±1.0	30 ±0.5	30 ±0.7
				R_{min}	14 ±1.5	14 ±1.2	0.0±0.0	0.0±0.0	0.0±0.0

pholipid preparations were examined by fluorescence polarization using DPH as the probe (Fig. 4). The transition temperature of DPPC has been well established at 41°C by a number of techniques including fluorescence polarization [29]. A sharp transition at 26°C was observed with 1-16:0,2-18:1-PE. The transition temperature of 16:0,2-18:1-PG was below 10°C (data not shown). Broader transitions were observed with the combined lipid systems. Addition of 1 mM $CaCl_2$ had no observable effect on the fluorescence polarization profile of DPPC/PE (5 : 5). The addition of 5 mM $CaCl_2$ to mixtures including PG resulted in a small but consistent sharpening of the profiles, particularly at the low temperature side of the curves (Fig. 4c, d).

Lipid aggregates were not observed when dispersion of DPPC/PE/PG (5 : 4 : 1) in saline-5 mM $CaCl_2$ were maintained at room temperature 24°C for 60 min (Fig. 3c). Incubation at 37°C for 30 min resulted in the formation of aggregated structures (Fig. 3d). Even greater aggregation was observed when a bubble was created and pulsated at 37°C (Fig. 3e). Surface tension measurements revealed that the appearance of the aggregated particles correlated with the development of good surfactant properties (Table II). The ability of these dispersions to reduce surface tension was not altered further by incubation at 37°C up to 4 h. These results are consistent with previous observations suggesting that below the hydrocarbon chain transition, mixed lipid systems prefer the bilayer structure. Above the transition temperature, the hydrocarbon chains become fluid, the cone-shaped character of PE is enhanced and an inverted micellar structure is favoured [20,30].

The effects of dispersing the lipid mixtures at various temperatures before incubation at 37°C on the surfactant activity are summarized in Table III. When the lipid mixtures were dispersed below the hydrocarbon chain transition temperature the formation of lipid aggregates was observed during incubation at 37°C and the mixtures exhibited surface tension reducing properties similar to natural surfactant. This was particularly evident with mixtures of DPPC/PE/PG (5 : 4 : 1) in the presence of 5 mM $CaCl_2$. Optimal surfactant activities were observed with mixtures disperesed just below 26°C, the transition temperature of the PE component (Fig. 4a). This observation corroborates the suggestion that the formation of lipid aggregates and the development of surfactant activity is related to the ability of PE to promote the transition from bilayer to nonbilayer structures. The inability of mixtures dispersed at low temperatures such as 4°C to form lipid aggregates and active surfactants is not understood but could be related to the lack of thorough intermixing of the components. The appearance of the aggregated lipid was significantly reduced when the components were dispersed above the PE phase transitions. This indicates that when the mixtures are dispersed in a fluid

TABLE III

EFFECT OF MIXING TEMPERATURE ON SURFACTANT ACTIVITY

Phospholipid samples (1%, w/v) were mixed at various temperatures for 1 h and then incubated at 37°C for 1–2 h before assaying as indicated in the text (mean ± S.E., $n = 4$).

Composition (%)			Parameter	Surface tension (dyn/cm)						
DPPC	PE	PG		4°C	10°C	15°C	20°C	24°C	30°C	37°C
50 (0.9% NaCl, 1 mM $CaCl_2$)	50		R_{max}	28 ±1.0	28 ±0.9	28 ±0.8	28 ±1.0	28 ±0.8	39±2.0	40±2.4
			R_{min}	8 ±0.6	7 ±1.0	0.0±0.0	1.0±1.0	1 ±0.6	16±1.5	20±1.7
50 (0.9% NaCl, 5 mM $CaCl_2$)	40	10	R_{max}	30 ±1.2	29 ±0.6	29 ±0.4	29 ±1.0	29 ±0.6	38±1.8	39±2.0
			R_{min}	4.0±0.8	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	10±1.0	10±1.4
60 (0.9% NaCl, 5 mM $CaCl_2$)	30	10	R_{max}	38 ±2.1	38 ±1.0	28 ±0.6	30 ±0.8	30 ±1.0	36±1.2	38±1.8
			R_{min}	14 ±1.0	6.0±0.6	4.0±0.8	0.0±0.0	0.0±0.0	12±1.1	14±1.6

form (Fig. 4c), a more stable bilayer structure is produced. With all phospholipids except PE, liposomes are more readily formed at temperatures producing fluid chains [31]. Hence, dispersing the lipid components at 30 °C and 37 °C probably enhanced the formation of stable closed liposomes.

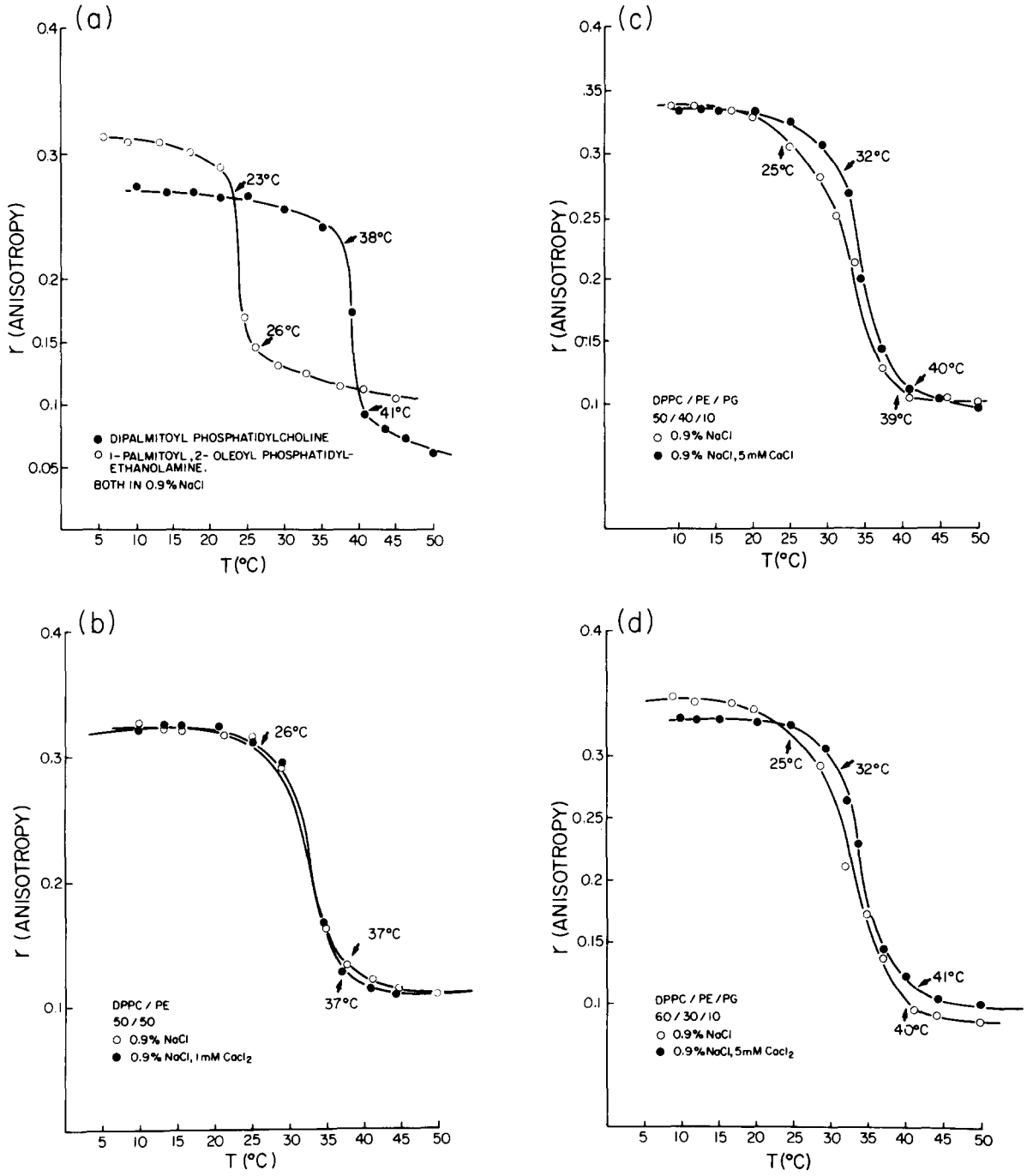

Fig. 4. Gel to liquid crystal transition profiles for single and mixed synthetic phospholipid samples as determined by fluorescent polarization using DPH as the probe.

TABLE IV

EFFECT OF pH ON SURFACTANT ACTIVITY OF MIXED PHOSPHOLIPID SYSTEMS ($n = 4$)

Composition (%)	pH	Surface tension (dyn/cm)	
		R_{max}	R_{min}
DPPC/PE	6.0	28±1.0	0.0±0.0
50:50	6.5	29±0.5	0.0±0.0
(0.9% NaCl,	7.0	28±0.6	0.0±0.0
1 mM $CaCl_2$,	7.5	30±1.0	4.0±0.4
5.8 mM $NaHCO_3$)	8.0	30±0.6	8.0±0.9
DPPC/PE/PG	6.0	29±0.5	0.0±0.0
50:40:10	6.5	29±0.9	0.0±0.0
(0.9% NaCl,	7.0	28±1.0	0.0±0.0
5 mM $CaCl_2$,	7.5	30±0.8	2.0±0.8
5.8 mM $NaHCO_3$)	8.0	30±1.0	4.0±1.0

Mechanical agitation may facilitate hydration and hence favour the formation of stable liposomes at fluidizing temperatures [31]. Under these circumstances, the bilayer-forming capacity of DPPC appears to dominate the nature of the structures formed. With these conditions, PE may concentrate within the inner half of the closed liposomal bilayer [32,22]. The flaky appearance of PE dispersions and their surface tension reducing properties were similar regardless of the temperature during mixing.

Influence of pH

The effect of the pH of the dispersing medium on the surfactant activities of DPPC/PE and DPPC/PE/PG mixtures in the presence of 1 and 5 mM $CaCl_2$, respectively, are listed in Table IV. In both cases excellent artificial surfactants were obtained at pH values from 6.0 to 7.0 but the ability to reduce the surface tension of the pulsating bubble to 0 dyn/cm became progressively depressed at higher pH values. The formation of H_{II} phase by unsaturated PE is increasingly inhibited above pH 8.0 [28]. Charge neutralization appears to be required for the formation of H_{II} phase. For example, the neutral system PC/PE/cholesterol does not require Ca^{2+} [30], but this divalent cation promotes H_{II} phase formation with acidic systems such as PC/cardiolipin [24]. The present studies reveal a requirement for Ca^{2+} when PG is present with DPPG and PE.

The amino group of PE possesses a pK_a of 7.5 [35]; this lipid becomes partially deprotonated above this pH. This results in a net charge, and consequently a repulsion in the polar headgroup region so that the molecules tend to assume the cylindrical shape favouring the bilayer organization [20]. Harlos and Eibl [36] have observed that with 1,2-di-16:O-PE, H_{II} phase can be detected at pH 12 only in the presence of Ca^{2+}. Thus it should be possible to prepare surface active phospholipid dispersions above pH 8 by increasing the Ca^{2+} concentration.

Effect of mechanical agitation

The results in Table V demonstrate that mild sonication with a bath sonicator is sufficient to

TABLE V

EFFECT OF SONICATION ON SURFACE TENSION REDUCING PROPERTIES

Active samples were sonicated at 24°C for 10 min in a bath-type sonicator, then incubated at 37°C for 1–2 h before the assay was repeated (mean ± S.E., $n = 4$).

Composition (%)			Parameter	Surface tension (dyn/cm)	
DPPC	PE	PG		Before sonication	After sonication
0	100	0	R_{max}	22 ±0.4	21±0.5
(1% in 0.9% NaCl)			R_{min}	16 ±0.4	16±0.6
50	50	0	R_{max}	29 ±1.0	46±2.2
(1% in 0.9% NaCl, 1 mM $CaCl_2$)			R_{min}	0.0±0.0	20±1.6
50	40	10	R_{max}	28 ±0.6	40±2.8
(1% in 0.9% NaCl, 5 mM $CaCl_2$)			R_{min}	0.0±0.0	18±1.0

destroy the surfactant characteristics of previously active samples. Sonication of the mixed phospholipid systems for 10 min at room temperature also resulted in a marked depletion of the lipid particles (Fig. 3f). Incubation at 37 °C for 30 min did not restore the surfactant activity. Sonication of DPPG/PG mixtures also reduces the rate of lipid adsorption to the air/liquid interface [37]. Dispersions of pure 1-16:0,2-18:1-PE were not affected by bath sonication at either 24 °C or 37 °C.

The stability of the apparent nonbilayer structures was influenced by the relative content of PE. Vortexing did not affect the surfactant activity of preparations containing DPPC/PE (5 : 5). However a few bursts of vigorous vortexing at 37 °C was sufficient to irreversibly destroy the surfactant function and the visible indications of nonbilayer structures with DPPC/PE/PG (5 : 4 : 1) and even more so with DPPC/PE/PG (6 : 3 : 1).

Comparison between natural surfactant and PE-containing artificial surfactants

Pulmonary surfactants derived from a variety of species contains approx. 35% DPPC, 25% unsaturated PC's, 10% PG plus phosphatidylinositol, 5% PE, 5% other phospholipids, 5–10% neutral lipid and 10% protein [1–3,23]. The most striking discrepancy in the phospholipid compositions of natural surfactant and the artificial surfactants described here is the 10-fold greater requirement for PE with the synthetic preparations. This difference presumably accounts for the differences in physical structure (Figs. 3a, d). Bovine surfactant which has a high proportion of DPPC and unsaturated PC remains dispersed, presumably primarily in bilayer structures upon hydration at 37 °C while the PE-containing artificial surfactants formed aggregates apparently arising from nonbilayer hydrophobic interactions at 37 °C. Unlike natural surfactant, the artificial dispersions did not display satisfactory surfactant activity in the absence of lipid aggregates.

Despite the marked differences in composition and physical appearance, pulmonary surfactant and the artificial preparations displayed similar rates of adsorption when assayed with the pulsating bubble surfactometer (Fig. 2). In addition, a close parallel was observed between the concentrations of natural surfactant and DPPC/PE/PG (5 : 4 : 1) required to reduce the surface tension to less than 30 dyn/cm at R_{max} and to near zero at R_{min} (Table VI).

Pulmonary surfactant is synthesized on the endoplasmic reticulum of alveolar cells, Type II, formed into lamellar bodies and secreted into the alveolar subphase. In the presence of Ca^{2+}, lamellar bodies can be transformed into tubular myelin, a form which consists of stacks of hollow tubes

TABLE VI

COMPARISON OF SURFACE TENSION REDUCING PROPERTIES OF PULMONARY AND ARTIFICIAL SURFACTANTS ($n = 4$)

Concentrations are given as % (w/v).

Samples	Parameter	Surface tension (dyn/cm)			
		Concn: 1	0.75	0.5	0.25
Pulmonary surfactant (0.9% NaCl, 1 mM $CaCl_2$)	R_{max}	27 ±1.0	27 ±0.8	28 ±1.3	35 ±2.2
	R_{min}	0.0±0.0	0.0±0.0	0.0±0.0	8.0±1.0
DPPC/PE 50:50 (0.9% NaCl, 1 mM $CaCl_2$)	R_{max}	29 ±0.6	29 ±1.0	30 ±1.0	38 ±2.0
	R_{min}	0.0±0.0	2.0±0.6	15 ±1.0	10 ±1.2
DPPC/PE/PG 50:40:10 (0.9% NaCl, 5 mM $CaCl_2$)	R_{max}	38 ±0.5	28 ±1.0	29 ±1.2	34 ±2.0
	R_{min}	0.0±0.0	0.0±0.0	1.0±0.8	8 ±0.8
DPPC/PE/PG 60:30:10 (0.9% NaCl, 5 mM $CaCl_2$)	R_{max}	29 ±1.0	30 ±1.2	30 ±1.4	35 ±2.1
	R_{min}	0.0±0.0	0.0±0.0	1.0±1.0	10 ±0.9

composing a regular square lattice [1,38–40]. It is presently thought that tubular myelin is directly involved in the formation of the surface active monolayer [1,41]. This could occur if tubular myelin contained unstable bilayers or nonbilayer structures from which the individual lipids could spontaneously flow onto the air-liquid interface. The selective loss of lipid onto the monolayer could explain the relatively high protein content of tubular myelin compared to lamellar bodies [38]. However, the precise nature of the interactions between the lipids in tubular myelin with other lipids, with water and with surfactant apoproteins remains vague. At present, the presence of nonbilayer lipid structures in tubular myelin can only be assumed. The present investigations demonstrate that phospholipid preparations containing a high proportion of PE can function as artificial surfactants. The expression of surfactant activity correlates with the appearance of lipid aggregates indicative of hydrophobic aggregation. The formation of these hydrophobic aggregates results in a rapid spreading at the air/liquid interface. These lipid aggregates appear only under circumstances where H_{II} phase can be anticipated. However, although our experimental approach was based on this mechanism, more direct studies are required to demonstrate the presence of inverted structures or H_{II} phase. In addition, it should be noted that only a very small amount of the DPPC present needs to be adsorbed onto the ulsating bubble to generate the observed alterations in surface tension. Consequently, a large proportion of the dispersed lipids could be present in forms which are not capable of rapid adsorption to the air/liquid interface.

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