Investigation of phospholipids of the pulmonary extracellular lining by electron paramagnetic resonance

The effects of phosphatidylglycerol and unsaturated phosphatidylcholines on the fluidity of dipalmitoyl phosphatidylcholine

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The membranous structures of the pulmonary extracellular lining were removed from the lungs of rabbits by pulmonary lavage and isolated by differential centrifugation. This membranous fraction contained 93% of the total extracellular phospholipids present in lavage effluents and consisted of membranous vesicles, membrane fragments, tubular myelin and secreted lamellar bodies. The fraction was rich in phosphatidylcholine (79.4%) containing 85.2% palmitic acid in the 1-position and 57.4% palmitic acid in the 2-position. Phosphatidylglycerol was the next most abundant phospholipid, accounting for 9.4% of the total. E.p.r. spectra, obtained by using 5-doxylmethylstearate as a probe, showed that the extracellular phospholipids of the pulmonary lining were organized into structures which were much more fluid than erythrocyte-ghost membranes. The fluidity of phosphatidylcholine isolated from the membranous fraction was similar to that of the fraction itself, indicating that the minor phospholipids had very little influence on the fluidity of the major phospholipid. At physiological temperature, the fluidity of dipalmitoyl phosphatidylcholine was relatively low, but could be markedly increased by the presence of 1-palmitoyl-2-oleoyl phosphatidylcholine or phosphatidylglycerol (10%). Protein present in the extracellular phospholipid fraction did not affect the fluidity of the fraction. These studies indicate that the unsaturated phosphatidylcholines could play a major role in determining the fluidity of the important surface-tension-lowering phospholipids such as dipalmitoyl phosphatidylcholine.

In the absence of the pulmonary extracellular lining, the alveoli and distal airways become unstable and collapse. The pulmonary extracellular lining is therefore absolutely essential for the maintenance of normal pulmonary functions. The constituents of the extracellular lining that are primarily responsible for the prevention of lung

collapse are several surface-active phospholipids of which the major one is DPPC (Brown, 1964; Clements, 1967; Rooney *et al.*, 1977).

Abbreviations used: DPPC, dipalmitoyl phosphatidylcholine; BHT, 2,6-di-*t*-butyl-4-methylphenol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; DMS, 5-doxylmethylstearate; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine.

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The phospholipids of the extracellular lining are synthesized in the alveolar Type 2 cells, where they are stored before secretion in the form of cytoplasmic organelles known as lamellar bodies. The lamellar bodies are secreted by an exocrinic process in which the perilamellar membrane fuses with the plasma membrane of the Type 2 cell, and the contents of the lamellar bodies are extruded into the lumen of the alveolus (Bensch *et al.*, 1964; Hatasa & Nakamura, 1965; Askin & Kuhn, 1971). Events subsequent to secretion are obscure. A surface film of phospholipid is formed at the air/

extracellular-lining interface; however, the processes by which this surface film is achieved from the tightly packed, concentric, multilamellated membranes of the secreted lamellar bodies are not known. The phospholipids of the secreted lamellar bodies must spread rapidly to form a film at the air/extracellular-lining interface and reduce the surface tension to approximately zero when the surface is fully compressed (King & Clements, 1972; Goerke, 1974; Clements, 1977). However, DPPC, the major surface-active ingredient of the alveolar surface, cannot spread rapidly at an air/water interface at temperatures below its gel-to-liquid-crystalline transition (41.5°C) (Clements, 1977; Goerke, 1974). Therefore these two essential requirements of rapid spreading and attenuation of surface tension cannot be attributed directly to DPPC. Two hypotheses have been advanced to account for the difference between the modified behavior of DPPC at the lung lining and DPPC in its pure form. One hypothesis attributes the rapid spreading of DPPC in the extracellular lining to the presence of other lipids such as phosphatidylglycerol (Hallman & Gluck, 1976; Bangham *et al.*, 1979), and the other identifies the modifying influence as an apoprotein (King & MacBeth, 1979; Pattle, 1965; Goldston *et al.*, 1969; Sueishi *et al.*, 1977). The mechanisms through which secreted DPPC spreads at the air/cell interface and the factors that influence that process are obscure.

The behaviour of DPPC at temperatures above and below its gel-to-liquid-crystalline transition indicates that fluidity of the phospholipid is a major factor in determining its ability to spread at an air/water interface. It is possible that the modifying influences referred to above focus primarily on the fluidity of the DPPC. If it is assumed that the rapid spreading of phospholipid at the air/cell interface is determined by the fluidity of the secreted lamellar bodies from which the phospholipid is derived, then factors such as compositional changes that influence membrane fluidity should also influence surface-film formation. Unfortunately, the relationship between fluidity and surface-film formation is not known.

In the present study we have examined the effect of composition on the fluidity of DPPC and phospholipids derived from the extracellular lining as an initial approach to clarifying the role of fluidity in the functioning of pulmonary surfactant.

Experimental procedures

Chemicals

The chemicals used (with their sources in parentheses) were as follows: DPPC (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.); 1-

palmitoyl-2-oleoyl phosphatidylcholine (Sigma Chemical Co., St. Louis, MO, U.S.A.); glutaraldehyde (Ladd Research Industries, Burlington, VT, U.S.A.); paraformaldehyde, sodium cacodylate, uranyl acetate and lead citrate (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.); OsO₄ (Colonial Metals, Elkton, MD, U.S.A.); DMS (Syva Associates, Palo Alto, CA, U.S.A.).

Animals

Adult male rabbits of the New Zealand strain (Dutchland Laboratory Animals, Denver, PA, U.S.A.) weighing from 2 to 2.5 kg were used throughout. Rabbits were allowed free access to food and water.

Pulmonary lavage procedure

Rabbits were killed by injection of 3–4 ml of sodium pentobarbital solution (50 mg/ml) into the marginal ear vein. The lungs were removed and lavaged eight times with ice-cold iso-osmotic saline as previously described (Hook, 1978). Lavage effluents were placed on ice.

Fractionation of lavage effluents

Lavage effluents were centrifuged at 580 *g*_{av.} for 10 min to sediment the cells. The supernatant was then centrifuged at 178 000 *g*_{av.} for 60 min (Beckman L3-50 ultracentrifuge). The 178 000 *g*_{av.} supernatant was then removed without disturbing the sediment. The 178 000 *g*_{av.} sediment was termed 'membranous fraction' after its resuspension in iso-osmotic saline.

Determination of phospholipids and fatty acids

Routine determinations of phospholipids were carried out by using the extraction procedure of Folch *et al.* (1957), and the colorimetric method of Shin (1962) was used for the determination of phospholipid phosphorus.

The phospholipid composition of the 'membranous fraction' was determined as follows. Phospholipids were extracted by using the method of Rouser & Fleischer (1967) with 0.005% BHT in the extraction solvents as an antioxidant (Wren & Szczekepanowska, 1964). The extracts were washed with 0.034% (w/v) MgCl₂ solution and evaporated to dryness under N₂. The preparation of plates and the solvents used for the partial separation of phospholipids were those of Skipski *et al.* (1967). Plates were run with the solvent system acetone/light petroleum (b.p. 35–60°C) (1:3, v/v), followed by chloroform/methanol/acetic acid/water (130:60:9:2, by vol.). Regions containing PE, PG, PI and PS were scraped from the plates and extracted four times with methanol. Phospholipids were replated and developed in a third solvent system (Gray, 1967) of the following

composition: tetrahydrofuran/dimethoxymethane/methanol/2M-NH₃ (10:6:4:1, by vol.). Phospholipids were located with I₂ vapour and provisionally identified by using appropriate standards. Phospholipids were quantified by using the method of Bartlett (1959) for the measurement of phosphorus.

The fatty acid composition of the phosphatidylcholine was determined after methylation of the samples with 4% (v/v) H₂SO₄ in methanol. The reaction was carried out in sealed vials for 1 h at 100°C. Light-petroleum extracts containing the methyl esters were dried under a stream of N₂. The methyl esters were solubilized in carbon disulphide before analysis on a Varian (model 3700) gas chromatograph with a column of Silar-10C (10%, w/w) on 100/120 Gas Chrom Q (Applied Science Division, State College, PA, U.S.A.). Fatty acids were provisionally identified by using appropriate standards.

Fatty acid distributions in the 1- and 2-positions of PC were determined by using the method of Dutta *et al.* (1979). PG was isolated from the lavage effluents as described above. Its fatty acid composition was not measured.

Preparation of liposomes

Multilaminar liposomes were prepared from extracted phospholipids by the procedure of DeGeir *et al.* (1968). Chloroform solutions of phospholipid were added to round-bottomed glass tubes and the solvent removed under a stream of dry N₂. A 3–5 ml portion of 0.25M-sucrose was added together with a glass bead, and the liposomes were formed by rapid agitation of the mixture. Final liposomal concentrations were adjusted to 500 µg of phospholipid/ml.

Protein

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Electron microscopy

Lungs were inflated *in situ* and fixed by perfusion of 2% (v/v) glutaraldehyde/1% paraformaldehyde buffered with 0.1M-phosphate, pH 7.4, at 37°C, through the pulmonary artery. The pressure of the perfusion medium did not exceed 30 cm of water and the perfusion was maintained for about 5 min. The lungs were then removed intact, dissected free of extraneous tissues, and then sliced into thin (about 1 mm thick) sections. Cubes of lung tissue (about 1 mm³) were cut from the sections and placed in fixative for 4 h at 4°C. The blocks were postfixed overnight in 1.5% OsO₄ buffered with 0.1M-sodium cacodylate, pH 7.4, at 4°C. Treatment with uranyl acetate and dehydration in acetone was performed as described by

Williams (1977). The blocks were embedded in Epon 812 (Luft, 1961).

The membranous fraction, isolated from lavage effluents, was prepared for electron microscopy as follows. The fraction resuspended in iso-osmotic saline was added to an equal volume of 4% glutaraldehyde/2% paraformaldehyde buffered with 0.2M-phosphate, pH 7.4, at 4°C. After 4 h the suspensions were centrifuged at 25 500 *g*_{av.} for 10 min. The pellets were postfixed overnight in 1.5% OsO₄ buffered with 0.1M-sodium cacodylate, pH 7.4, at 5.4°C. Treatment with uranyl acetate and dehydration in acetone were performed as above by the method of Williams (1977).

Ultrathin sections were cut on a Porter–Blum MT-1 ultramicrotome with a du Pont (E. I. Du Pont de Nemours and Co., Wilmington, DE, U.S.A.) diamond knife. The sections were stained with 5% uranyl acetate in ethanol (Watson, 1958) for 10 min, then with lead citrate (Reynolds, 1963) for 6 min, and were examined in a Philips 300 electron microscope.

E.p.r.

The spin label (DMS) in methanolic solution was dried on to the sides of incubation tubes under a stream of N₂. Liposomes (250 µg of phospholipid) suspended in 0.25M-sucrose (0.5 ml) were added and incubated with the spin label (final concentration was 20 µM) for 10 min at room temperature. The samples were then placed in the spectrometer and allowed to equilibrate at 37°C before scans were initiated. E.p.r. spectra were recorded with a Varian E-109 X-Band spectrometer equipped with a TM₁₁₀ cavity and a variable-temperature accessory. The temperature was monitored continuously with a specially constructed probe that fitted into the flat region of the quartz aqueous-sample flat cell. The probe was therefore in direct contact with the sample at all times. The effect of temperature on the motion of the intercalated spin label was determined by measuring the relative amplitudes of the central peak (B) and the low-field peak (A). Instrumental parameters were as follows: scan range 10 mT; field set, 0.33 T; time constant, 0.5 s; scan time, 4 min; modulation amplitude, 0.4 mT; modulation frequency, 100 KHz; receiver gain, 2.0 × 10⁴; microwave power, 30 mW; and microwave frequency, 9.25 GHz.

Results

Membranous structures of the extracellular lining

By using differential centrifugation we isolated from pulmonary lavage effluents a fraction that contained 93% of the total extracellular phospho-

lipids (Table 1). This fraction contained only 34% of the total extracellular proteins. Since most of the extracellular phospholipid was contained in this fraction, we assume that we have isolated most of the phospholipid-rich structured entities from which the surface film is derived, as well as fragments of the surface film itself. The electron microscope revealed that the fraction consisted primarily of membranous vesicles, membrane fragments, tubular myelin, and secreted lamellar bodies (Fig. 1*a*). All of the extracellular structures found in the lavage effluents were also present in the extracellular lining of the lungs. Fig. 1(*b*) shows

the extracellular lining overlying a Type 2 cell in the alveolus of a rabbit lung. Secreted lamellar bodies, membranous vesicles, tubular myelin and the surface film are all evident. The surface film appears as an osmiophilic line at the air/lining interface (Fig. 1*c*). Some regions of the surface film appear thicker than usual, as shown in Fig. 1(*d*), indicating, perhaps, piling up of the surface film. Although considerable heterogeneity of structure exists, the fraction that we have isolated from lavage effluents is clearly representative of the structured materials present in the extracellular lining and, in addition, contains most of the

Table 1. *Distribution of phospholipid and protein between fractions of lavage effluents from the lungs of rabbits*
The lungs of a rabbit were lavaged eight times with iso-osmotic saline as described in the Experimental procedures section. Cells were sedimented at 580*g*_{av.} for 10 min. The membranous fraction was sedimented at 178 000*g*_{av.} for 1 h. The 'soluble phase' was the 178 000*g*_{av.} supernatant. Results are means ± S.D. (*n* = 4).

	Phospholipid		Protein	
	(mg)	(%)	(mg)	(%)
Total lavage	30.7 ± 7.5	100	27.2 ± 1.4	100
Cells	5.3 ± 2.0	17.1 ± 3.2	11.4 ± 1.1	41.9 ± 3.0
Soluble phase	1.6 ± 0.2	5.3 ± 1.2	11.8 ± 1.1	43.3 ± 4.2
Membranous fraction	22.3 ± 3.9	73.9 ± 8.3	3.8 ± 1.0	13.9 ± 3.6

Fig. 1. *Structured elements of the pulmonary extracellular lining*

A, Membranous fraction of lavage effluents from the lungs of rabbits. The fraction consisted of membranous vesicles (MV), membrane fragments (MF), tubular myelin (TM), and secreted lamellar bodies (SLB). Magnification × 13900. B, Electron micrograph of the air/tissue interface showing the extracellular lining overlying an alveolar Type 2 cell. The surface film (SF) is continuous with tubular myelin and secreted lamellar bodies, suggesting that tubular myelin may be an intermediate between secreted lamellar bodies and the surface film. Membranous vesicles are also present. Magnification × 20700. C, Enlargement of the extracellular lining showing the surface film and underlying hypophase (HP). Magnification × 89300. D, Surface film of the extracellular lining showing thickened regions. These thickened regions may arise from piling up of the surface film. Magnification × 89300.

extracellular phospholipid. We will refer to this fraction as the ‘membranous fraction’.

The phospholipid composition of the membranous fraction is shown in Table 2. The major phospholipid was PC (79.4%); PG was the second most abundant, and accounted for 9.4% of the total phospholipid. Similar compositions have been reported by others (Rooney *et al.*, 1977; Jobe *et al.*, 1978) for preparations of pulmonary-surfactant-derived phospholipids. The fatty acid composition of the PC present in the membranous fraction is shown in Table 3. Palmitic acid was the major fatty acid, accounting for 85.2% of the total fatty acid

present in the 1-position and 57.4% in the 2-position.

This fatty acid composition is similar to that reported by others for surfactant-related PC (Frosolono *et al.*, 1970, 1971; Jobe *et al.*, 1978). However, the maximum percentage of DPPC that could exist in the phosphatidylcholine of the extracellular lining is only about 57%. Since unsaturated fatty acids account for approx. 30% of the fatty acids in the 2-position, but only 9% in the 1-position, at least 20% of the phosphatidylcholine must be present with unsaturation in the 2-position and saturation in the 1-position.

E.p.r.

Previous studies have shown that DMS is a useful probe for monitoring the physical state of phospholipid membranes (Cannon *et al.*, 1975). The e.p.r. spectrum of DMS in the membranous fraction of lavage effluents at 37°C (Fig. 2) revealed that the probe was quite mobile, suggesting that the phospholipids were in a relatively fluid state. However, the e.p.r. spectrum of DMS in DPPC liposomes (Fig. 2) was characteristic of a probe whose motion is more constrained. Some

Table 2. Phospholipid composition of the membranous fraction of pulmonary lavage effluents

Lavage effluents from the lungs of eight rabbits were pooled and fractionated as described in the Experimental Procedures Section. The membranous fraction was analysed for phospholipids by t.l.c. Results are means ± s.d. (n = 3). Further abbreviations used: lyso-PC, lysophosphatidylcholine; Sph, sphingomyelin.

Phospholipid	Phospholipid (% of total)
Lyso-PC	2.2 ± 0.1
Sph	0.9 ± 0.3
PC	79.4 ± 3.2
PI	0.7 ± 0.2
PS	1.0 ± 0.3
PG	9.4 ± 2.0
PE	6.5 ± 1.4

Table 3. Fatty acid composition of phosphatidylcholine from the membranous fraction of lavage effluents

PC was isolated from the fraction of lavage effluents from the lungs of eight rabbits by the procedures described in the Experimental Procedures section. Fatty acid distribution between the 1- and 2-positions of the PC was determined by the methods of Dutta *et al.* (1979). Results are means ± s.d. (n = 5). Abbreviation used: ND, not detected.

Fatty acid	Distribution (%)	
	1-Position	2-Position
C ₁₄	2.1 ± 0.9	7.3 ± 0.9
C _{14:1}	1.1 ± 0.2	10.6 ± 1.6
C ₁₆	85.2 ± 3.6	57.4 ± 3.4
C _{16:1}	0.8 ± 0.4	3.0 ± 0.6
C ₁₈	3.8 ± 1.1	0.4 ± 1.2
C _{18:1}	2.1 ± 0.8	1.9 ± 0.7
C _{18:2}	1.7 ± 0.6	6.9 ± 0.6
C _{18:3}	0.8 ± 0.2	0.1 ± 0.2
C ₂₀	0.7 ± 0.2	5.0 ± 0.4
C _{20:1}	0.5 ± 0.1	ND
C _{20:2}	0.5 ± 0.1	7.5 ± 0.1
C _{20:3}	0.9 ± 0.4	ND
C _{20:4}	0.2 ± 0.1	ND

Fig. 2. E.p.r. spectra of DMS in the membranous fraction (MF) of pulmonary lavage effluents, liposomes of pure DPPC and liposomes of PC isolated from the membranous fraction.

Spectra were obtained at 37°C. Note the close similarity of spectra obtained from membranous fragments and PC from membranous fragments. The spectrum of DMS in DPPC indicates that the probe is highly restrained.

difference must therefore exist between the physical states of the phospholipids in the membranous fraction and DPPC liposomes, and it may well be that it is this difference which enables phospholipids of the membranous fraction to act as a rapidly spreading pulmonary surfactant. It is of interest to note that the spectrum of DMS in PC isolated from the membranous fraction was very similar to that of the intact membranous fraction itself, despite its high DPPC content. Thus it would appear that the influence of the non-PC phospholipids on the fluidity of the PC present in the membranous fraction must be quite small.

It has been proposed that PG, which, in our membranous fraction, accounted for $9.4 \pm 2.0\%$ of the total phospholipid, acts as a modifier of DPPC to facilitate its function as the pulmonary surfactant (Hallman & Gluck, 1976; Bangham *et al.*, 1979). The influence of this minor phospholipid on the fluidity of DPPC vesicles is shown in Fig. 3. The spectrum of DMS in pure PG indicates that the liposomes are highly fluid and that the influence of PG on the fluidity of DPPC is substantial. The ratio of the amplitude of the centre-field peak to the low-field peak (B/A) is inversely related to the mobility of DMS, such that a value of unity was approached when DMS is free in solution (Stuhne-Sekalec & Stanacev, 1977b). Although this ratio is empirical, it does provide a

convenient method for the comparison of the mobility of DMS in different environments. The ratio B/A varied according to the concentration of PG in DPPC (Fig. 3) and at concentrations above 10% the mobility of the probe was greater than that seen in the membranous fraction itself. At 9.4% PG the B/A ratio (1.58) was similar to that found in the membranous fraction (1.59). Thus it would appear that natural PG can substantially influence the fluidity of DPPC.

The fluidity of DPPC as measured with DMS is also markedly affected by the presence of unsaturated PC. As shown in Fig. 4, the fluidity of POPC is much greater than that of DPPC at 37°C. The influence of POPC on DPPC is considerable and, as found with PG, the B/A ratio varied with the concentration of POPC. However, the B/A ratio (1.62) of pure POPC was very similar to that of the membranous fraction (1.59). According to our analysis, as much as 20% of the PC present in the membranous fraction could be PC with an unsaturated fatty acid in the 2-position and a saturated fatty acid in the 1-position. A concentration of 20% POPC in DPPC would give a B/A ratio of about 1.70, which lies close to that of the membranous fraction (1.59). The increase in the fluidity of DPPC on addition of POPC is not due to the preference of the probe for the unsaturated component, since the probe will distribute between

Fig. 3. E.p.r. spectra of DMS in synthetic DPPC, PG (from cell-free lavage effluents) and in various concentrations of PG in DPPC.

The ratio of B to A was measured from the spectra as shown and was used as a relative measure of membrane fluidity. Complete molecular freedom of the probe would give rise to a B/A ratio of 1. The spectra were obtained at 37°C. The graph demonstrates the dependency of the B/A ratio on the concentration of PG in DPPC. Each point is the average result for four or five determinations. Standard deviations were generally less than 10% of the mean. MF indicates 'membranous fraction'. From the top the spectra are for pure DPPC, 1% PG in DPPC, 4% PG in DPPC, 30% PG in DPPC, and pure PG.

Fig. 4. *E.p.r. spectra of DMS in synthetic DPPC and synthetic POPC and various concentrations of POPC in DPPC*. The spectra were obtained at 37°C. The ratio of B to A was measured from the spectra as shown and was used as a relative measure of membrane fluidity. Complete molecular freedom of the probe would give rise to a B/A ratio of 1. The graph demonstrates the dependency of the B/A ratio on the concentration of POPC in DPPC. Each point is the average of two or three determinations. MF indicates 'membranous fraction'. From the top the spectra are for pure DPPC, 1% POPC in DPPC, 5% POPC in DPPC, 10% POPC in DPPC, and pure POPC.

both species of PC regardless of how the probe is added to the mixture (Fig. 5). If DMS is added to the POPC/DPPC mixture, the spectrum obtained is identical with that produced when the probe is premixed with POPC and then added to DPPC.

Temperature

The influence of temperature on the fluidity of DPPC liposomes is shown in Fig. 6. DPPC liposomes showed a distinct transition at 41°C, below which there was a marked decrease in fluidity. Below 40°C the membranous fraction was much more fluid than DPPC. The fluidity of PC liposomes isolated from the membranous fraction was also much greater than those of DPPC at temperatures below 40°C. Above 40°C the fluidity of PC liposomes isolated from the membranous fraction and those of DPPC were very similar. The membranous fraction showed a distinct break at 38°C, but with PC from the membranous fraction the break was at 41°C, indicating that the minor phospholipids of the membranous fraction may have influenced the temperature at which the break occurred; however, because the discontinuities are determined from the placement of the curves, some uncertainty exists as to the precise temperature of the break-point.

Below 40°C, POPC was much more fluid than DPPC (Fig. 6). Addition of 10% POPC to the DPPC markedly changed the response of the B/A ratio to temperature. The fluidity characteristics, as a function of temperature, of DPPC containing 10% POPC resembled those of the membranous fraction and pure POPC. Clearly, POPC can markedly alter the fluidity of DPPC at temperatures below 40°C. Above 40°C the fluidity of DPPC, POPC, and the membranous fraction were similar.

Phosphatidylglycerol was more fluid than DPPC and the membranous fraction at all temperatures studied (Fig. 7). Concentrations of PG in DPPC below 5% did not markedly alter the fluidity-temperature characteristics of the DPPC. At 10% PG the alterations in B/A ratio as a function of temperature were shown to be similar to those of the membranous fraction. The curve for 10% PG in DPPC was calculated from the family of curves generated on plotting percentage of PG against the B/A ratio for each temperature below 40°C.

The influence of protein on fluidity was studied by comparing the membranous fraction with liposomes prepared from chloroform/methanol (2:1, v/v) extracts of the membranous fraction.

Fig. 5. E.p.r. spectra of DMS in liposomes of synthetic DPPC containing 10% synthetic POPC.

(a) DMS was added to liposomes of POPC, mixed thoroughly, and then added to liposomes of DPPC. (b) Liposomes were prepared from a mixture of DPPC and POPC followed by the addition of DMS. The similarity of the spectra indicates that DMS will freely distribute between DPPC and POPC. Spectra were obtained at 37°C.

Because chloroform/methanol effectively extracted all of the lipids but very little protein (Folch *et al.*, 1957; Stein & Smith, 1982), the major compositional difference between the liposomes and the membranous fraction lay with the proteins. The B/A ratios of the membranous fraction were similar to that of the liposomes at all temperatures studied (results not shown), indicating that protein constituents had very little influence on the fluidity of lipids in the membranous fraction. The ability of protein to decrease markedly the fluidity of membrane lipids in our system was confirmed by comparing erythrocyte-ghost membranes with liposomes prepared from those ghosts. At all temperatures studied, the B/A ratio was much higher for erythrocyte ghosts than for liposomes without the protein constituents (results not shown) (e.g., at 37°C, B/A = 2.3 for erythrocyte ghosts, 2.15 for liposomes from erythrocyte ghosts and 1.6 for the membranous fraction). The restraining influence of proteins on the mobility of DMS in erythrocyte ghosts has also been reported by others (Benga & Chapman, 1976; Benga *et al.*, 1979; Chapman *et al.*, 1979).

Discussion

The pulmonary extracellular lining exists as a duplex consisting of a relatively thick basal layer in contact with the cells of the pulmonary epithelium and a very thin superficial layer interposed

Fig. 6. Effect of POPC on the temperature variation of the B/A ratio

The B/A ratio was measured, as indicated in Fig. 3, from e.p.r. spectra of DMS in aqueous suspensions of liposomes of synthetic DPPC, membranous fraction (MF), liposomes of PC isolated from the membranous fraction (MFPC), liposomes of synthetic POPC, and liposomes of mixtures of POPC and DPPC. POPC and DPPC were mixed before preparation of the liposomes.

between the air and the basal layer (Weibel & Gil, 1968; Gil & Weibel, 1969/1970; Kikkawa, 1970; Manabe, 1979). The basal and superficial layer are known as the hypophase and surface film respectively. Under the electron microscope the hypophase generally appears structureless, consisting of amorphous material; however, regions may be found containing highly organized materials such as tubular myelin, secreted lamellar bodies, and a wide variety of membranous vesicles (Weibel & Gil, 1968; Stratton, 1977). The surface film, which appears osmiophilic under the electron microscope, is the crucial location at which surface-tension forces act to collapse the lungs (Clements, 1977) if not prevented from doing so by surface-active phospholipids. Our studies indicate that the

Fig. 7. Effect of PG on the temperature variation of the B/A ratio

The B/A ratio was measured, as indicated in Fig. 3, from e.p.r. spectra of DMS in aqueous suspensions of liposomes of synthetic DPPC, natural PG and mixtures of PG in DPPC. PG and DPPC were mixed before preparation of liposomes. Abbreviation used: MF, membranous fraction.

major phospholipids of pulmonary lavage effluents are associated with easily sedimented membranous structures and that these structures were probably the structural elements of the hypophase of the extracellular lining. However, we cannot discount formation of at least some of the membranous structures from phospholipids present in the extracellular lining as a result of the lavage and isolation procedure. Although the relationship between individual structures is uncertain, the membranous vesicles are probably derived from secreted lamellar bodies and represent various stages in the dispersal of phospholipids from the bodies. The role of tubular myelin in the sequence between secretion of lamellar bodies and formation of the surface film is not known.

The use of hydrophobic spin-labelled molecules to study the organization of membrane components is well known; however, little use of the method has been made to study the pulmonary-surfactant system of the lungs. The long hydrophobic tail of DMS favours intercalation of the molecule into the hydrophobic regions of membranes with a probe alignment similar to that of the fatty acid acyl chains of the membrane phospholipids.

Discontinuities in the fluidity-temperature relationships of pure phospholipids in aqueous dispersion are generally indicative of phase transitions. For pure DPPC the gel-to-liquid-crystalline phase transition occurs at 41.5°C, as determined by several different methods including one employing DMS (Stuhn-Sekalec & Stanacev, 1977a). However, all of the lipid systems studied, including pure POPC, showed sharp discontinuities in their

fluidity-temperature relationships around 40°C, indicating that all of the observed breaks may not be due to the same phase transition experienced by pure DPPC at this temperature. Pure POPC would not be expected to undergo its gel-to-liquid-crystalline phase transition at the same temperature as pure DPPC. The observed discontinuities occurred at temperatures similar to those reported by Stuhn-Sekalec & Stanacev (1977a), who also used several probes with the spin label in the 5-position of the acyl groups. These investigators attributed the discontinuities to co-operative interactions between polar head groups of the phospholipids and methylene groups in the near vicinity. These interactions allowed motional freedom of methylene groups near the polar head groups to approach that of a crystalline hydrocarbon, whereas, in the same chain, freedom near the terminal methyl group approached that of a liquid hydrocarbon. If this interpretation is correct, then DMS with the spin label in the 5-position reported the motional freedom of the acyl chains of the phospholipids near the polar head groups. Thus pulmonary-surfactant phospholipids show a sharp alteration in motional freedom of methylene groups near their polar head groups around physiological temperatures. This transition is not affected by surfactant-associated proteins, but is influenced by the minor phospholipids.

The dispersal of phospholipids from secreted lamellar bodies and the formation of a stable surface film requires that the membranes of the lamellar bodies be highly fluid. The major phospholipid of the extracellular lining is DPPC; however, below its gel-to-liquid-crystalline transition temperature of 41.5°C, pure DPPC will not spread spontaneously at an air/water interface (Clements, 1977; Bangham *et al.*, 1979). Clearly, the minor components of the secreted lamellar bodies must play a major role in modifying the fluidity characteristics of DPPC.

Our studies demonstrate that the fluidity characteristics of the complex mixture of phospholipids present in the extracellular lining are very similar to those of its major component, phosphatidylcholine. Since the fluidity characteristics of PC isolated from surfactant resembled those of surfactant, the modifying influence must reside with the unsaturated phosphatidylcholines. In support of this contention we have demonstrated a marked fluidity-enhancing effect of POPC on DPPC. The minor phospholipids appear to have relatively little influence on the fluidity of the total surfactant mixture, although, as we have shown, PG from the extracellular lining is capable of modifying the fluidity characteristics of DPPC to the extent that a mixture of the two resembled the fluidity characteristics of surfactant. It is possible that a

role for PG in surfactant might be to lower the transition temperature of DPPC. However, our data are not sufficient to establish this point with certainty, and other studies will be required. Bangham *et al.* (1979) also contend that the DPPC-modifying influence resides with another lipid and demonstrated that a mixture of DPPC and unsaturated PG (30%) mimicked many of the properties of natural surfactant. Our results are not strictly comparable, since different properties were investigated.

In recent years, several investigators have concluded that proteins do not play a role in stabilization of the alveoli and distal airways (Stein, 1969; Shelley *et al.*, 1975; Metcalfe *et al.*, 1980). Our data tend to support these conclusions insofar as protein did not influence the fluidity characteristics of the membranous structures of the extracellular lining; however, we cannot exclude the possibility that protein may contribute to formation of the surface film, as indicated by King & McBeth (1979) and Meban (1981).

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