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STRUCTURAL STUDIES ON LAMELLATED OSMIOPHILIC BODIES ISOLATED FROM PIG LUNG

^{31}P NMR RESULTS AND WATER CONTENT

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Summary

1. Lamellated osmiophilic bodies are intracellular organelles in which pulmonary surfactant is stored prior to secretion. They contain about 85% phospholipid (per dry weight) and dipalmitoyl phosphatidylcholine is a major constituent, and although their ultrastructure is uncertain it is generally supposed that they resemble liposomes. However, liposomes are stable because layers of water are interposed between the lipid bilayers whereas an essential aspect of the function of lamellated bodies is that, subsequent to their secretion, they are rapidly disrupted to form a surface-active film which covers the respiratory epithelium of the lung.

2. A new method for isolating lamellated bodies from pig lung is described which has the advantage of speed and simplicity and which results in increased yields. The homogeneity of the preparation is similar to that obtained by other methods.

3. ^{31}P NMR spectra of lamellated bodies showed that at 40°C about 95% of the phospholipid was present as extended bilayers and that about 5% was present in a phase exhibiting isotropic head group mobility ($\tau_R < 10^{-5}$ s). It is suggested that this phase may be due to apolar proteins which are present both in lamellated bodies and in liposomes prepared from lipids extracted from them.

4. The internal water content of lamellated bodies has been measured gravimetrically and the hydration of the phospholipid head groups has been exam-

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ined by ^{31}P NMR. The two methods gave results in good agreement and show that there are about seven molecules of water/molecule of phospholipid. It is concluded that although the phospholipid head groups in lamellated bodies are fully hydrated, there is no zone of free water.

5. Lamellated bodies are osmotically insensitive to NaCl whereas liposomes prepared from lipids extracted from them behave like perfect osmometers. It is suggested that the osmotic insensitivity and restricted water content of lamellated bodies are important to their function and dependent upon polar proteins in the outer limiting membrane.

Introduction

Pulmonary surfactant reduces surface tension at the alveolar surface and is essential for normal lung function [1,2]. It is synthesized by Type 2 cells in the lung and is stored in lamellated osmiophilic bodies [3] prior to secretion. These organelles contain about 85% phospholipid, of which dipalmitoyl phosphatidylcholine is the main constituent and phosphatidylglycerol is an important minor component. The ultrastructurure of lamellated bodies is controversial, and whereas some investigators find no distinctive internal architecture, others by either electron microscopy or by freeze-fracture find concentric laminations although the reported dimensions vary considerably [4].

In the presence of excess aqueous phase, phospholipids form liposomes in which lipid bilayers are separated by layers of water [5]. However, liposomes are extremely stable [6], in contrast to lamellated bodies which rapidly disrupt to form a surface-active film following their secretion by Type 2 cells. The structural basis for this difference in behaviour is unknown and since it may depend on phospholipid hydration [7] we have used ^{31}P NMR to investigate the organization and hydration of the phospholipids in lamellated osmiophilic bodies isolated from pig lung and we have measured their internal water content by gravimetric methods.

Methods and Materials

All sucrose solutions contained 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA, and all operations were carried out at 0—5° C. Pig lung was transported to the laboratory on ice and 200 g was removed from the periphery, cut into cubes with sides of about 2 cm and homogenized in 400 ml 0.3 M sucrose in a Waring blendor with six bursts of 10 s at top speed. The homogenate was centrifuged for 5 min at $700 \times g$ and the supernatant filtered through eight layers of surgical gauze. The sucrose content of the filtrate was adjusted to 0.75 M by adding 2 M sucrose and 30-ml aliquots were transferred to Beckman rotor 21 tubes and successively overlaid with 40 ml 0.65 M sucrose and 20 ml 0.3 M sucrose and centrifuged for 3 h at $55\,000 \times g_{max}$. The lamellated body fraction which formed a band at the interface between 0.65 M sucrose and 0.3 M sucrose was removed, diluted to 0.3 M sucrose by dropwise addition of 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, and centrifuged for 1 h at $106\,000 \times g_{max}$. The pellet of lamellated bodies was used immediately for the experiments

described below. The yield was 13.4 ± 2.5 (S.E.) μg phospholipid phosphorus/g wet wt of lung.

Estimation of internal water content of lamellated bodies. Lamellated bodies (about 200 μg of phospholipid phosphorus) were gently suspended with a loose fitting Teflon-in-glass hand homogenizer in 5 ml 0.3 M sucrose containing either 1 mM sorbitol and 5 μCi D-[U-^{14}C]sorbitol, or 5 μCi [U-^{14}C]sucrose (Radiochemical Centre, Amersham). The suspension was centrifuged for 1 h at 170 000 × g_{max} in a Beckmann SW 39L rotor. The upper 3 ml of the supernatant was discarded and four aliquots each of 0.2 ml of the remaining supernatant were transferred to tared scintillation vials which were then reweighed. A further sample of supernatant was removed with a tared calibrated 250-μl constriction pipette for specific gravity determination. The remaining supernatant was decanted and the time noted and the moist pellet rapidly transferred to a small tared glass weighting dish. The weight of the dish and pellet was measured on a Mettler 5M microbalance (Mettler Instrumente AG CH-8606, Greifensee-Zurich) to the nearest μg at intervals of 1 min for 5 min and the initial wet weight of the pellet calculated by extrapolation to zero time. The dish was then covered with a loose fitting glass lid and dried at 50 Pa over P_2O_5 for 2 days at 70°C and reweighed. The total water content of the pellet was determined by difference. Control experiments showed that drying was complete under the conditions described. The dried pellet was extracted successively with 10 portions each of 5 ml of chloroform/methanol/2 M NH_4OH (18 : 9 : 1, v/v) and it was shown that this procedure completely removed radioactivity from the pellet. The extracts were pooled, made up to volume and four tared aliquots, each of 5 ml, was used for radioactivity measurement. The extraparticulate water content of the pellet was calculated on the assumption that its specific radioactivity and specific gravity were equal to that of supernatant since control experiments showed no evidence of sedimentation of either radioactive sucrose or sorbitol under the conditions described. The weight of intraparticulate water was determined by subtracting the extraparticulate water from the total water content of the pellet.

^{31}P NMR spectroscopy. ^{31}P NMR spectra were recorded on a Pulse FT spectrometer built in Oxford equipped with either a 7.5 or 4.3 Tesla magnet using a 50° pulse, an interpulse time of 0.5 s and accumulating up to 1000 free induction decays. Lamellated bodies were suspended in 0.3 M sucrose containing 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA. Liposomes were prepared and suspended in the same medium by the method of Bangham [8] using lipids extracted from lamellated bodies by the method of Bligh and Dyer [9]. The effects of varying hydration on lamellated body lipids were investigated by depositing them as a film on the wall of the sample tube which was then dried as described previously. Known proportions of water were then added and the tube was sealed in an atmosphere of N_2 and equilibrated for at least 10 h at 50°C.

Radioactivity measurements. Vials contained 1 ml water and 9 ml Aquasol and radioactivity was measured in an LKB-Wallac 81000 liquid scintillation counter using the sample channels ratio method. In all cases sample size was determined gravimetrically and all estimations were carried out in quadruplicate. The mean value of the coefficient of variation between quadruplicate samples was 0.5% (range 0.1—1.2%).

Other methods. Continuous sucrose gradients were prepared by the method of Noll [10]. Protein was estimated in the presence of 0.5% sodium dodecyl sulphate by the method of Lowry et al. [11] using bovine plasma albumin (Armour Pharmaceutical Co. Ltd. Eastbourne, England) as a standard, or by amino acid analysis following hydrolysis in 6 M HCl for 18 h at 105° C using a JLC-6AH amino acid analyser (Jeolco, Tokyo, Japan). Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on plates 0.25 mm thick by the method of Poorthuis et al. [12] and visualized with I_2 vapour. Phosphorus analysis was carried out by the method of Bartlett [13], cholesterol by a modification of the method of Huang et al. [14], and glycerides by the method of Wahlefeld [15].

Results

Composition and homogeneity of lamellated body fraction

The lipid composition of the lamellated body fraction is given in Table I. The fatty acids of the phosphatidylcholine isolated by two-dimensional TLC were analysed by gas-liquid chromatography (GLC) and contained 84% palmitate. The lamellated bodies contained 76 μg protein/μmol of phospholipid phosphorus when protein was estimated by the method of Lowry et al. [11] but by amino acid analysis the total protein content was 41 μg/μmol of phospholipid phosphorus. Lipid extracts prepared from lamellated bodies contained 11 μg protein/μmol of phospholipid phosphorus as estimated by amino acid analysis. Electron microscopy showed that lamellated bodies possessed an outer limiting membrane and were between 0.5 and 1.0 μm in diameter. Only very small quantities of amorphous material were seen. The homogeneity of the preparation was further investigated by suspension of the pellet, obtained as described in Methods and Materials, in 0.75 M sucrose which was then overlaid

TABLE I

COMPOSITION OF LAMELLATED BODIES ISOLATED FROM PIG LUNG

Lamellated bodies were isolated and analysed as described in Methods and Materials. The protein content of intact lamellated bodies and of the lipid extract prepared from them was estimated by amino analysis.

	Phospholipid composition (expressed as percentage of total phospholipid) (%)	Other constituents (expressed per μmol of phospholipid phosphorus)
Phosphatidylcholine	78.6	
Phosphatidylethanolamine	7.1	
Phosphatidylserine + phosphatidylinositol	7.1	
Phosphatidylglycerol	3.0	
Sphingomyelin	1.6	
Lysophosphatidylcholine	0.8	
Unidentified	2.6	
Total cholesterol		0.14 μmol
Glycerides		0.08 μmol
Total protein		41 μg
Protein in lipid extract		11 μg

TABLE II

INTERNAL WATER CONTENT OF LAMELLATED BODIES

Lamellated bodies were equilibrated in the presence of either [U-^{14}C]sorbitol or [U-^{14}C]sucrose and after centrifugation were analysed as described in Methods and Materials. Results are expressed in μmol.

	Radioactive marker					
	[U-^{14}C]Sorbitol			[U-^{14}C]Sucrose		
Experiment No.	1	2	3	4	5	6
Phospholipid	2.05	2.79	4.26	3.97	3.00	3.89
Total water	1239	1651	2129	2165	1445	1993
Extra particulate water	1226	1633	2068	2142	1439	1969
Internal water	13	18	61	23	6	24
μmol internal water per μmol phospholipid	6.2	6.3	14.2	5.9	2.1	6.1

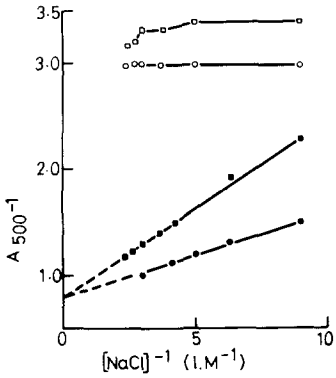

Fig. 1. Osmotic behaviour of lamellated bodies and liposomes. Lamellated bodies, or liposomes prepared from them were suspended in 0.3 M sucrose containing 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA. A_{500nm} was measured on a Cary 14 recording spectrophotometer and aliquots of 2 M NaCl in 0.3 M sucrose/Tris-EDTA were added and A_{500nm} measured after 15 min equilibration. Observations were made on lamellated bodies at 22° C (○) and 37° C (□) and on liposomes at 22° C (●) and 37° C (■).

with a continuous sucrose density gradient extending from 0.75 M sucrose to 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA and centrifuged in a Beckman SW 40 rotor at 284 000 $\times g_{max}$ for 18 h at 5° C. A single band, mean density 1.060 g/cm^3 was obtained.

Internal water content of lamellated bodies

The results of six experiments using either [U-^{14}C]sucrose or [U-^{14}C]sorbitol as a marker for extraparticulate water are given in Table II. The total water content of the pellet of lamellated bodies was 82% of the initial wet weight (range 81.6—82.4%) but virtually all of this was due to extraparticulate

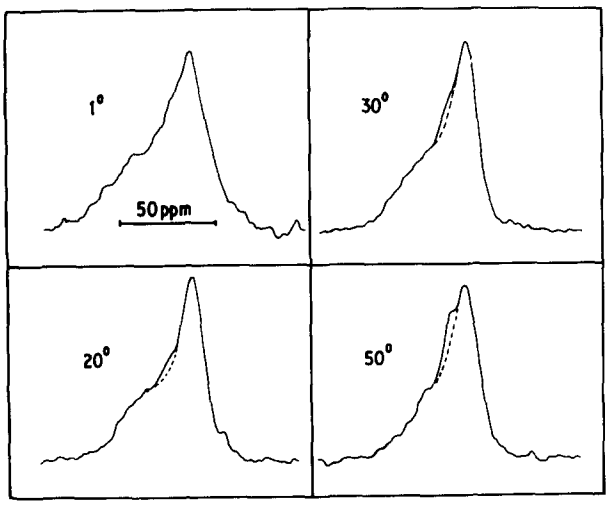

Fig. 2. ^{31}P NMR spectra of lamellated bodies suspended in 0.3 M sucrose containing 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA. The spectra were recorded at 129 MHz without proton decoupling.

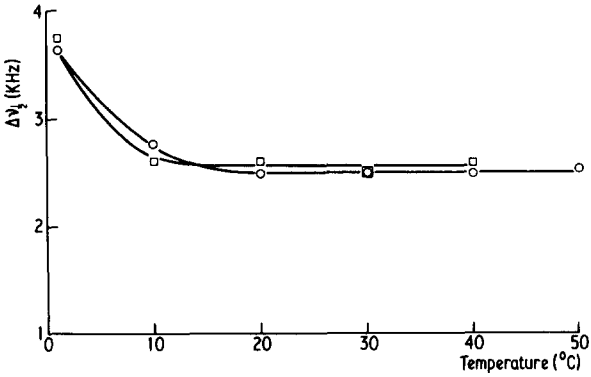

Fig. 3. Temperature dependence of the line width at half height $\Delta\nu_{1/2}$ of the 129 MHz ^{31}P NMR spectra obtained from lamellated bodies (○) and liposomes (□) prepared from lipids extracted from lamellated bodies.

medium. The mean value for the internal water content of the lamellated bodies was 7 ± 1.5 (S.E.) mol of water/mol of phospholipid phosphorus.

Osmotic behaviour of lamellated bodies and of liposomes extracted from them

Fig. 1 shows that as assessed by the reciprocal of A_{500nm} [16] the volume of liposomes was decreased by increasing NaCl concentration and that over the concentration range studied they behaved in accordance with the Boyle-van't Hoff relationship $V = k/c + V_{c\to\infty}$ where V is volume, k is a constant, c is salt concentration, and $V_{c\to\infty}$ is the osmotic dead space. In contrast, lamellated bodies were only slightly affected and under the experimental conditions described they appeared osmotically insensitive.

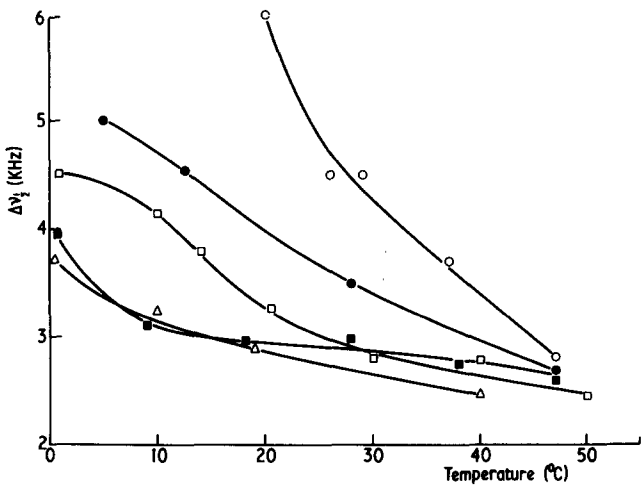

Fig. 4. Effect of different degrees of hydration on the phase transition temperature of lipids extracted from lamellated bodies. Lipids were extracted and hydrated as described in Methods and Materials. ^{31}P NMR spectra were recorded at 73.8 MHz without proton decoupling and the linewidth at half height is plotted against temperature when the molar ratio of water to phospholipid was 1 (○), 6 (●), 6.5 (□), 8 (△) and >12 (■).

^{31}P NMR spectroscopy

Spectra of lamellated bodies recorded at 129.2 MHz are shown in Fig. 2 and it can be seen that as the temperature increased a narrow peak developed on the low field shoulder and the spectrum became markedly narrower. Both these changes were reversible. The spectra of liposomes prepared from lamellated body lipids were identical and as shown in Fig. 3 the temperature dependence of the line width at half peak height for both lamellated bodies and liposomes showed that a phase transition occurred at about 15°C. The spectra in Fig. 4 were recorded at 73.8 MHz and it is apparent that when lipids extracted from lamellated bodies were equilibrated with varying proportions of water, the temperature at which the phase transition occurred fell with increasing hydration, and reached a limiting value of about 15°C when the water content was between 6 and 8 mol of water/mol of phospholipid.

Discussion

The method used for the isolation of lamellated bodies differs from those previously described [17,18] in that they were separated from the crude homogenate in a single step by upward centrifugation through a discontinuous sucrose density gradient. The main advantage was that yields were considerably increased and since isolated lamellated bodies are unstable [19] this may be a consequence of the speed and simplicity of the procedure. The purity of the lamellar body fraction as assessed by lipid composition, electron microscopy and homogeneity on continuous density gradient centrifugation was similar to that obtained with other methods.

The ^{31}P NMR spectra of lamellated bodies were indistinguishable from those of liposomes prepared from lipids which had been extracted from them. In both cases the spectra were characteristic of extended phospholipid bilayers [20] with the exception of a narrow peak which became more prominent on the low field shoulder as the temperature increased. The position and relative area of this peak implies that at 40°C about 5% of the total phospholipid was present in a phase with isotropic head group mobility ($\tau_R < 10^{-5}$ s), e.g. as inverted micelles, short hexagonal pipes or as a cubic phase [21,22]. It is unlikely that this is due to the presence of small vesicles because its appearance was readily reversible with temperature and although mixtures of phosphatidylethanolamine and phosphatidylcholine permit the formation of structures showing isotropic spectra when the proportion of phosphatidylethanolamine exceeds 50% [21], only about 7% was present in the lipids of lamellated bodies (Table I). It is therefore possible that this phase is induced by the apolar proteins which were present both in lamellated bodies and in lipid extracts prepared from them (Table I).

The decrease in line width with temperature (Figs. 1 and 2) is due to increased mobility of the phospholipid head groups consequent upon a liquid crystalline to gel phase transition in the fatty acyl side chains [23,24]. It is known that this transition is sensitive to water content [25] and the finding that in both lamellated bodies and liposomes the transition occurred at about 15°C implied that in both cases the hydration of the phospholipid head groups was similar. The gravimetric estimation of the internal water content of

lamellated bodies showed however that they contained only about 15% water whereas liposomes contain up to 50% [7] and we have also shown that lamellated bodies differ from liposomes in that they are osmotically insensitive. The relationship between the head group mobility of phospholipids extracted from lamellated bodies and water content was therefore investigated. It should be noted that in these experiments, the lipid was deposited as a film on the inner surface of the sample tube and in consequence the averaging of dipolar interactions was reduced which results in line broadening. On the other hand the field strength was lower and hence signal spread due to chemical shift anisotropy was diminished. Thus, although the absolute values of the line widths are not directly comparable with previous experiments, Fig. 3 shows that diminution with increasing hydration became maximal and insensitive to further addition of water when the water content was about 15%. It is apparent therefore that the gravimetric estimation of the water content of isolated lamellated bodies is consistent with the data obtained from ^{31}P NMR spectroscopy.

Small [26] calculated from X-ray diffraction studies of phosphatidylcholine/water mixtures that about eight molecules of water could be accommodated in the phospholipid head group region and that water in excess of this was located between the lipid bilayers. Taken together with the results reported above this suggests that although lamellated bodies resemble liposomes in that the phospholipids are predominantly present as extended bilayers and the head groups are fully hydrated, they differ in that in lamellated bodies there is no zone of free water. This conclusion is consistent with the ultrastructural studies of Stratton [27] who examined lamellated bodies in situ in Type 2 cells in lung using techniques designed to minimize lipid extraction and found a lamellar repeat distance of 66 Å and the absence of an interlamellar space. It should also be noted that the absence of a free water zone in lamellated bodies is likely to be of functional significance since it has been found that dry sheep surfactant is more effective than surfactant liposomes in forming surface-active films and in enhancing lung inflation in the immature rabbit foetus [28].

Finally, the experiments reported above show that the proteins of lamellated bodies have different locations and functions. The main difference in composition between lamellated bodies and liposomes prepared from them is that the latter are devoid of proteins which are insoluble in organic solvents and in consequence it is concluded that the absence of a free water zone and the osmotic insensitivity of lamellated bodies is dependent upon their presence and this in turn suggests that these proteins are located in the outer limiting membrane. On the other hand, evidence has been presented that the lipid-soluble proteins in lamellated bodies are responsible for the presence of an isotropic phospholipid phase. Preliminary estimates of the molecular weights of these proteins (Phizackerley, P.J.R. and Town, M.-H., unpublished) suggested that between 10 and 100 molecules of phospholipid may be associated with each protein molecule. It is of interest in this connection that freeze-fracture studies show the presence of particles and ridges on the outer surface of the lamellae [29]. The function of these structures is not known but it is possible that they are involved either in the assembly of lamellated bodies or in the formation of tubular myelin [30] following their secretion.

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