Hydrophobic Proteins of Lamellated Osmiophilic Bodies Isolated from Pig Lung

By Patrick J. R. PHIZACKERLEY, Michael-Harold TOWN and Geoffrey E. NEWMAN
Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford OX2 6HE, U.K.

(Received 29 May 1979)

1. In addition to proteins that are insoluble in organic solvents, lamellated bodies isolated from pig lung and surfactant prepared from bronchopulmonary lavage fluid contain another group of proteins that are extracted together with lipid into the organic phase. 2. These hydrophobic proteins constitute about 40% of the total protein of lamellated bodies and about 13% of the total protein of surfactant isolated from lavage fluid, whereas less than 1% of the total protein of pig lung microsomal fraction and mitochondria is extracted by organic solvents. 3. The hydrophobic proteins of lamellated bodies were separated into four fractions and freed from phospholipid by chromatographic procedures. Their apparent molecular weights vary between 11 500 and 16 500, they contain 72–79% of hydrophobic residues and 16–22% of sulphur-containing amino-acids, and leucine is the major *N*-terminal amino acid in each case.

Pulmonary surfactant lowers tension at the alveolar surface and in consequence decreases the work of ventilation and prevents alveolar collapse in expiration. It is synthesized by type II cells in the lung and is stored in lamellated osmiophilic bodies before secretion. These organelles are about 1 μ m in diameter and are bounded by an outer limiting membrane. Lipids comprise 80–90% by weight of both lamellated bodies and of surfactant prepared from bronchopulmonary lavage fluid, and it has been shown in a wide variety of mammalian species that dipalmitoyl phosphatidylcholine is the main constituent and phosphatidylglycerol is an important minor component (for reviews see Pattle, 1965; Goerke, 1974; King, 1974). In contrast, the nature of the proteins associated with surfactant is uncertain, and depends not only on the methods used to isolate surfactant (Abrams, 1966; Klein & Margolis, 1968; Pruitt *et al.*, 1971; King & Clements, 1972*a,b*; Gil & Reiss, 1973; King *et al.*, 1973, 1977; Scarpelli *et al.*, 1973; Bhattacharyya *et al.*, 1975; Shelley *et al.*, 1975; Sawada & Kashiwamata, 1977; Sawada *et al.*, 1977) but also on species (Harwood *et al.*, 1975).

Morley *et al.* (1978) have shown that dry surfactant is more effective than surfactant liposomes in lowering surface tension, and in consequence they suggested that the capacity of the phospholipids of lamellated bodies to form a surface-active film was dependent on their limited hydration. It has been shown in this laboratory (Grathwohl *et al.*, 1979) by ^{31}P n.m.r. spectroscopy and other methods that lamellated bodies from pig lung are osmotically insensitive, that their internal water is restricted to the phospholipid head-group region and that these properties are dependent on proteins in their outer limiting membrane that were insoluble in organic

solvents. It was also shown that, although about 95% of the phospholipid in lamellated bodies is present as extended bilayers, about 5% is present as a phase exhibiting isotropic head-group mobility ($\tau_R < 10^{-5}$ s), and evidence was obtained which suggested that this phase was dependent on hydrophobic proteins that were extracted together with lipids by organic solvents. These proteins, which are also present in surfactant isolated from bronchopulmonary lavage fluid, have not been described previously, and the purpose of the present paper is to report a method for their isolation and some of their properties.

Methods

Preparation of lamellated bodies from pig lung and extraction with organic solvents

Lamellated bodies were prepared from 200 g of pig lung as described previously (Grathwohl *et al.*, 1979), resuspended in 10 ml of water, and sucrose was removed by dialysis against several changes of water at 4°C. The contents of the dialysis sac were freeze-dried and stored at –20°C. For the experiments reported below, preparations from 3 kg of pig lung were pooled and extracted five times by vortex mixing for 1 min at room temperature with diethyl ether/ethanol (3:1, v/v) (1 ml of solvent/mg of sample). After each extraction the suspension was centrifuged at 700 g for 30 min at 4°C and the supernatants were combined. The extract was evaporated to dryness and the residue was dissolved in 10 ml of chloroform. It contained 800 mg of phospholipid and 29 mg of protein.

Preparation of surfactant from bronchopulmonary lavage fluid

Pig lung lower lobe was lavaged five times with 30 ml of 0.15 M-NaCl via the bronchus, and surfactant was isolated from the lavage fluid by a procedure identical with that used to prepare lamellated bodies from whole lung.

Preparation of mitochondria and microsomal fraction

All sucrose solutions contained 10 mM-Tris/HCl buffer, pH 7.4, and 0.1 mM-EDTA, and all operations were carried out at 0–5°C. A 100 g portion of lung was cut from the periphery into cubes with sides of about 2 cm and homogenized in 200 ml of 0.3 M-sucrose in a Waring Blendor with six bursts of 10 s at top speed. The homogenate was centrifuged at 700 g for 5 min and the supernatant was filtered through eight layers of surgical gauze. For the preparation of mitochondria, the filtrate was centrifuged at 1000 g for 10 min and the pellet was discarded. The supernatant was layered on to 0.5 vol. of 0.65 M-sucrose and centrifuged at 12000 g for 15 min. The pellet constituted the mitochondrial fraction. For the preparation of microsomal fraction the filtrate obtained as described above was centrifuged at 12000 g for 15 min and the pellet was discarded. The supernatant was layered on to 0.5 vol. of 0.65 M-sucrose and centrifuged at 106000 g_{max} for 2 h in a Beckman SW 25.2 rotor. The pellet constituted the microsomal fraction.

Silicic acid column chromatography

Silicic acid (100 mesh; A.R. grade; Mallinckrodt, St. Louis, MO, U.S.A.) was washed several times with excess of chloroform and methanol, activated by heating at 110°C for 15 h, suspended in chloroform and transferred to a column (20 cm × 3 cm diam.) (1 g of silicic acid/25 mg of phospholipid). The sample in chloroform was applied to the column, which was eluted under gravity with 500 ml of chloroform, 360 ml of chloroform/methanol (20:1, v/v), 540 ml of chloroform/methanol (9:1, v/v), 360 ml of chloroform/methanol (4:1, v/v), 360 ml of chloroform/methanol (3:2, v/v), 360 ml of chloroform/methanol (1:4, v/v) and finally with 360 ml of 50% (v/v) acetic acid. The chloroform and acetic acid eluates were collected in bulk, and all other eluates were collected in 10 ml fractions. The fractions were monitored by determination of P by the method of Bartlett (1959), by determination of amino groups both before and after acid hydrolysis (with AristaR 6M-HCl at 105°C for 18 h), by the fluorescamine procedure (Udenfriend *et al.*, 1972) and by the qualitative Sakaguchi reaction for arginine (Jepson & Smith, 1953). The phospholipid composition of selected fractions was examined by two-dimensional t.l.c. by the method of Poorthuis *et al.* (1976).

Separation of protein from phospholipid by column chromatography on Bio-Gel P-160

Silicic acid column fractions containing protein were pooled, and samples containing about 300 µg of protein were evaporated to dryness and dissolved in 1 ml of phenol/acetic acid/water (1:1:1, w/v/v) to which had been added 5 drops of glycerol. The sample was applied to a column (100 cm × 1 cm diam.) of Bio-Gel P-100 equilibrated in the same solvent and eluted under gravity. Fractions (1 ml) were collected and monitored by determination of P and of amino groups as described above. Fractions containing protein were pooled and separated from phenol by column chromatography on Bio-Gel P-2. This column was 12 cm × 2 cm diam. and was equilibrated and eluted with 50% (v/v) acetic acid. Fractions (1 ml) were collected and monitored by determination of amino groups and measurement of the absorbance of phenol at 288 nm.

*Determination of protein by the method of Lowry *et al.* (1951)*

This determination was carried in the presence of 0.5% (w/v) sodium dodecyl sulphate, with bovine serum albumin (Sigma Chemical Co., Poole, Dorset, U.K.) as standard.

Amino acid analysis

Samples were hydrolysed in an atmosphere of N₂ for 18 h at 105°C in AristaR 6M-HCl, with and without pretreatment by the performic acid oxidation procedure (Hirs, 1967). Amino acid analysis was carried out on a JEOL 6AH amino acid analyser (JEOL Co., Tokyo, Japan), with norleucine and homoarginine as internal standards.

Identification of N-terminal amino acids

The method of Gray (1967) was used, and after acid hydrolysis samples were chromatographed in two dimensions on 5 cm × 5 cm polyamide layer sheets (Cheng-Chin Trading Co., Taipeh, Taiwan), and authentic dansyl-amino acids were chromatographed on the reverse side. The first-dimension solvent was 1.5% (w/v) formic acid and the second dimension was run successively in toluene/acetic acid (9:1, v/v) and ethyl acetate/methanol/acetic acid (20:1:1, by vol.). Dansyl derivatives were located by u.v. light.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was carried out as described by Weber & Osborn (1969), with 5% acrylamide gels and 0.1 M-sodium phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulphate as electrophoresis and gel buffer.

Results

Solubility of proteins of lamellated bodies in organic solvents

Table 1 shows that, as determined by the method of Lowry *et al.* (1951), about 96% of the total protein of lamellated bodies was apparently recovered in the residue insoluble in ether/ethanol, and protein was not detected in the lipid extract. However, when protein was determined by amino acid analysis after acid hydrolysis, it is apparent that only about 60% of the total protein of lamellated bodies was insoluble in ether/ethanol, and the remaining 40% was extracted together with lipid in the organic phase (Table 2). Similar results were obtained when lipids were extracted with chloroform/methanol (2:1, v/v) by the procedure of Folch *et al.* (1957) and when 10 mM-benzamidine, 1 mM-EDTA, 1 mM-*o*-phenanthroline, 0.1 mM-4-chloromercuribenzoate or pancreatic trypsin inhibitor (0.5 mg/ml) was added to the lung homogenate from which the lamellated bodies were isolated in order to inhibit proteolysis. When pulmonary surfactant isolated from bronchopulmonary lavage fluid was extracted with ether/

ethanol about 13% of the total protein was recovered in the lipid extract, but with lung mitochondria and microsomal fraction the corresponding value was less than 1% (Table 2).

Column chromatography on silicic acid of lipid extract of lamellated bodies

The elution procedure was designed to separate proteins rather than phospholipids, and the results are shown in Fig. 1. Four protein fractions were obtained, designated A-D, and, although chloroform/methanol mixtures eluted all the phospholipid applied to the column, 35% of the protein was retained. This protein was eluted with 50% acetic acid and, since it was shown by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to consist of at least four proteins that were similar to those present in the ether/ethanol-insoluble fraction, it was not examined further.

Separation of hydrophobic proteins of lamellated bodies from phospholipid

Tubes containing fraction A and tubes containing the protein peaks B, C and D were pooled, and the amino acid compositions of these fractions are given in Table 3. It was shown by t.l.c. that phosphatidylcholine was present in all fractions and that, in addition, phosphatidylethanolamine and phosphatidylserine were present in fraction A and phosphatidylethanolamine in fraction B. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis all four fractions showed a diffuse band, migrating just ahead of the dye front (mol. wt. < 20000), that contained both protein and phospholipid, and pilot experiments showed that protein was extracted together with phospholipid by hexane, ether, chloroform, methanol and ethanol. However, as shown in Fig. 2, protein was successfully separated from phospholipid by column chromatography on Bio-Gel P-100 in phenol/acetic acid/water (1:1:1, w/v/v), and in all

Table 1. *Comparison between the total protein and ether/ethanol-insoluble protein content of lamellated bodies as determined by the method of Lowry et al. (1951)*

Experimental details are given in the text. The protein content of lamellated bodies, and of the residue remaining after extraction with ether/ethanol, was determined by the method of Lowry *et al.* (1951) in the presence of 0.5% sodium dodecyl sulphate, with bovine serum albumin as standard.

Expt. no.	Lamellated body protein (μg of protein/100 μg of phospholipid)	
	Before extraction	After extraction
1	8.3	8.1
2	8.6	8.1
3	7.8	7.5

Table 2. *Solubility in ether/ethanol of proteins of pig lung lamellated bodies, of surfactant isolated from bronchopulmonary lavage fluid and of pig lung mitochondria and microsomal fraction determined by amino acid analysis*

Subcellular fractions prepared from pig lung and surfactant isolated from bronchopulmonary lavage fluid were extracted with ether/ethanol (3:1, v/v) as described in the Methods section. The protein contents of the insoluble residue and of the extract were measured by amino acid analysis after hydrolysis in 6M-HCl at 105°C for 18 h. Results for lamellated bodies are expressed as means $\pm$ S.E.M. for the numbers of determinations given in parentheses; in other cases, results are expressed as means $\pm$ range.

Fraction	Protein (μg /100 μg of phospholipid)		Soluble proteins (% of total protein)
	Insoluble protein	Soluble protein	
Lamellated bodies	4.5 $\pm$ 0.3 (8)	2.9 $\pm$ 0.4 (4)	39
Surfactant	4.2 $\pm$ 0.3 (2)	0.68 $\pm$ 0.02 (2)	12.7
Mitochondria	349 $\pm$ 15 (2)	3.1 $\pm$ 0.2 (2)	0.9
Microsomal fraction	284 $\pm$ 27 (2)	0.65 $\pm$ 0.05 (2)	0.2

Fig. 1. Silicic acid column chromatography of lipid extract of lamellated bodies

Fractions were monitored by determination of total P (○) and of amino groups (●) before and after acid hydrolysis in 6M-HCl at 105°C for 18h.

cases protein was recovered as a single peak. This procedure also permits the determination of molecular weight (Pusztai & Watt, 1970), and the values recorded in Table 3 were obtained in this way.

N-Terminal amino acids of hydrophobic proteins

In order to identify the *N*-terminal amino acids of proteins A–D it was first necessary to remove phenol, and this was achieved by gel filtration on Bio-Gel P-2 in 50% acetic acid. The most prominent dansyl-amino acid in all four proteins was leucine. In the case of protein C, dansyl-leucine was the only dansylated amino acid present, but traces of other dansyl amino acids were detected in the other chromatograms.

Amino acid composition of protein C

The quantities of proteins A, B and D that remained after the purification procedures described above

were insufficient for accurate analysis, but the amino acid composition of protein C was determined, and, on the basis of mol.wt. 16 500 (Table 3), the residues/molecule were Asp 6, Lys 6, Arg 9, Thr 5, Ser 3, Pro 12, Gly 14, Ala 8, Val 11, Ile 11, Leu 32, Tyr 2, Phe 3, Cys 23 and Met 5.

Discussion

The experiments reported above show that, in addition to proteins that remained as a residue after extraction with organic solvents, both lamellated bodies isolated from pig lung and surfactant prepared from bronchopulmonary lavage fluid contained a second group of proteins that were extracted together with lipid into the organic phase. This extract gave no reaction for protein by the procedure of Lowry *et al.* (1951), and in confirmation of the observations made by King *et al.* (1973), as estimated by this method, the total protein content of lamellated bodies was identical, within experimental error, with the protein content of the residue that remains after

Table 3. Amino acid composition of protein fractions A–D obtained by silicic acid column chromatography of lipid extract of lamellated bodies

The protein fractions were obtained as described in the Methods section and as shown in Fig. 1. Amino acid analyses were carried out with and without performic acid oxidation by the method of Hirs (1967). The values for cysteine and for methionine were derived from the first of these procedures and for tyrosine from the second. All other values are mean values, and tryptophan was not determined. Fraction A contained phosphatidylserine, and in column A(1) the results are calculated on the assumption that serine is derived solely from protein, and in column A(2) on the assumption that it is derived solely from phospholipid.

Protein fraction Apparent mol.wt.	Amino acid composition (residues/1000 residues)				
	A		B	C	D
	16000	11500	16500	16500	16500
	(1)	(2)			
Hydrophilic amino acids					
Asp	49.4	56.6	48.6	50.1	51.8
Glu	30.9	35.4	10.7	24.8	35.1
Lys	38.5	44.1	39.2	52.4	60.4
His	3.6	4.1	6.1	11.6	1.9
Arg	53.5	61.3	68.2	78.2	59.0
Thr	17.9	20.5	7.9	11.2	25.7
Ser	127.3	—	27.8	20.2	43.3
Hydrophobic amino acids					
Pro	117.5	134.6	116.2	63.9	87.7
Gly	59.6	68.3	57.3	76.0	66.0
Ala	47.3	54.2	41.7	55.4	73.2
Val	56.5	64.9	57.5	70.0	90.1
Ile	46.3	53.1	67.3	57.5	57.1
Leu	158.4	181.5	219.9	221.6	149.1
Tyr	4.8	5.5	3.0	6.7	14.3
Phe	8.2	9.4	7.6	12.8	26.7
Cys	150.7	172.7	178.9	150.4	138.5
Met	29.3	33.6	42.4	37.3	20.3

Fig. 2. Separation of protein C from phospholipid by column chromatography on Bio-Gel P-100

Fractions obtained by silicic acid column chromatography that contained protein C were pooled, dissolved in phenol/acetic acid/water (1:1:1, w/v/v) and chromatographed in the same solvent on Bio-Gel P-100 as described in the Methods section. Fractions were monitored by the determination of amino groups. Phospholipid was absent from the first peak and was quantitatively recovered in the second peak. The results obtained with the other protein fractions were similar.

extraction with ether/ethanol. However, two-dimensional t.l.c. of lipid extracts of both lamellated bodies and of lavage surfactant showed an origin spot that gave a positive reaction to ninhydrin and to the Sakaguchi reagents for arginine, and amino acid analysis of the extracts after acid hydrolysis conclusively demonstrated the presence of protein. It seems likely that the failure to detect these proteins by the procedure of Lowry *et al.* (1951) is partly a consequence of their low content of tyrosine and partly because access of the Lowry reagents is prevented by lipids.

The finding that only a very small proportion of the total protein of other subcellular fractions prepared from pig lung was soluble in ether/ethanol shows that these proteins are distinctive components of lamellated bodies. They are difficult to separate from phospholipids, and in their presence they migrate together on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and are soluble in many organic solvents. The most striking feature of their composition is that all four protein fractions contain 16–22% of sulphur-containing amino acids, mainly 'cysteine + $\frac{1}{2}$ cystine', and 72–79% of hydro-

phobic residues, especially leucine, which is the major *N*-terminal amino acid in all cases.

The similarity in composition of these proteins suggests that, whatever their specific functions may be, they may also have functions in common. As pointed out above, there is evidence from ^{31}P n.m.r. spectroscopy that the hydrophobic proteins of lamellated bodies may interact with phospholipid head groups to form an isotropic phase, and in this context it is relevant that both high-resolution electron microscopy (Stratton, 1976*a,b*) and freeze-fracture studies of lamellated bodies (Untersee *et al.*, 1971; Smith *et al.*, 1972) show the presence of numerous particles of about 10 nm diameter on the outer surface of the internal lipid lamellae of lamellated bodies. The structure of these particles is not known, but it seems possible that they consist of the hydrophobic proteins described above, arranged together with phospholipid as inverted micelles with their non-polar residues exposed to the hydrocarbon lipid phase. The disruption of such a structure would be expected to occur on exposure to the aqueous environment of the alveolar subphase and could play a part in the complex events that follow the secretion of lamellated bodies. These events, which are initiated by the loss of the outer limiting membrane of lamellated bodies by fusion with the apical plasma membrane of the type II cell (Ryan *et al.*, 1975), lead to the rapid formation of tubular myelin (Weibel *et al.*, 1966; Stratton, 1977*a,b*) and of a surface-active film at the alveolar interface. Further studies on the structure of these hydrophobic proteins may help to resolve these problems and may also be relevant to the question of whether the physiological properties of surfactant are due solely to their content of phospholipid.

M.-H. T. was the holder of a Medical Research Council Studentship for Training in Research Methods.

References

- Abrams, M. E. (1966) *J. Appl. Physiol.* **21**, 718–720
 Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
 Bhattacharyya, S. N., Passero, M. A., DiAugustine, R. P. & Linn, W. S. (1975) *J. Clin. Invest.* **55**, 914–920
 Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
 Gil, J. & Reiss, O. K. (1973) *J. Cell Biol.* **58**, 152–171
 Goerke, J. (1974) *Biochim. Biophys. Acta* **344**, 241–261
 Grathwohl, C. H., Newman, G. E., Phizackerley, P. J. R. & Town, M.-H. (1979) *Biochim. Biophys. Acta* **552**, 509–518
 Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–151
 Harwood, J. L., Desai, R., Hext, P., Tetley, T. & Richards, R. (1975) *Biochem. J.* **151**, 707–714
 Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 59–62
 Jepson, J. B. & Smith, I. (1953) *Nature (London)* **172**, 1100–1101
 King, R. J. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 2238–2247
 King, R. J. & Clements, J. A. (1972*a*) *Am. J. Physiol.* **223**, 707–714
 King, R. J. & Clements, J. A. (1972*b*) *Am. J. Physiol.* **223**, 715–726
 King, R. J., Klass, D. J., Gikas, E. G. & Clements, J. A. (1973) *Am. J. Physiol.* **224**, 788–795
 King, R. J., Martin, H., Mitts, D. & Holmstrom, F. M. (1977) *J. Appl. Physiol.* **42**, 483–491
 Klein, R. M. & Margolis, S. (1968) *J. Appl. Physiol.* **25**, 654–658
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Morley, C. J., Bangham, A. D., Johnson, P., Thorburn, G. D. & Jenkins, G. (1978) *Nature (London)* **271**, 162–163
 Pattle, R. E. (1965) *Physiol. Rev.* **45**, 48–79
 Poorthuis, B. J. H. M., Yazaki, P. J. & Hostetler, K. Y. (1976) *J. Lipid Res.* **17**, 433–437
 Pruitt, K. M., Cherng, M. J. & Spitzer, H. L. (1971) *Arch. Intern. Med.* **127**, 390–394
 Pusztai, A. & Watt, W. B. (1970) *Biochim. Biophys. Acta* **214**, 463–467
 Ryan, U. S., Ryan, J. W. & Smith, D. S. (1975) *Tissue Cell* **7**, 587–599
 Sawada, H. & Kashiwamata, S. (1977) *Biochim. Biophys. Acta* **490**, 44–50
 Sawada, H., Yamabayashi, H. & Okajima, Y. (1977) *Biochim. Biophys. Acta* **493**, 196–200
 Scarpelli, E. M., Wolfson, D. R. & Colacicco, G. (1973) *J. Appl. Physiol.* **34**, 750–753
 Shelley, S. A., L'Heureux, M. V. & Balis, J. U. (1975) *J. Lipid Res.* **16**, 224–234
 Smith, D. S., Smith, U. & Ryan, J. W. (1972) *Tissue Cell* **4**, 457–468
 Stratton, C. J. (1976*a*) *Tissue Cell* **8**, 693–712
 Stratton, C. J. (1976*b*) *Tissue Cell* **8**, 713–728
 Stratton, C. J. (1977*a*) *Tissue Cell* **9**, 285–300
 Stratton, C. J. (1977*b*) *Tissue Cell* **9**, 301–316
 Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Loimgruber, W. & Weigle, M. (1972) *Science* **178**, 871–872
 Untersee, P., Gil, J. & Weibel, E. R. (1971) *Respir. Physiol.* **13**, 171–185
 Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
 Weibel, E. R., Kister, E. S. & Tondury, G. (1966) *Z. Zellforsch.* **69**, 418–427