ISOLATION AND CHARACTERIZATION OF LUNG SURFACTANT

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It is well established that saline extracts of lungs contain a surfactant which reduces surface tension when spread at an air-water interface. This reduction is augmented by compression of the interface, and is believed to play a physiological role by preventing alveolar collapse. However, the composition of lung surfactant remains controversial. Extraction with organic solvents has shown that phospholipids are major components (Brown et al., 1964). The identity of components other than lipids, or whether non-lipid material is present at all, has not been adequately established. Generally surfactant is assumed to be a lipoprotein (Abrams, 1966) but recent evidence suggests that polysaccharides may be associated with the lipids (Scarpelli et al., 1967).

The difficulties have arisen largely because of inadequate methods for isolation and purification. An approach must be devised which permits the isolation of surfactant uncontaminated by extraneous materials, without employing organic solvents or other drastic techniques. By using centrifugation, and by monitoring the procedure with surface activity determinations and chemical analysis, we have isolated from dogs a material which fulfills the operational definition of surfactant established by other workers. The

surfactant is a mixture of lipids; the sum of protein and carbohydrate content is less than 3%.

ISOLATION OF SURFACTANT

Healthy mongrel dogs under light pentobarbital anesthesia were sacrificed by exsanguination. The excised lungs were collapsed in a vacuum chamber and washed repeatedly by distending them via the tracheobronchial tree with 0.15M NaCl and collecting the liquid by passive recoil of the lung. Turbidity measurements of each lavage correlated with dry weight of purified surfactant and indicated that 80% was recovered in the first two washes. Virtually all the remainder was recovered by the sixth lavage. Beginning with the crude saline extract, the fractions obtained during the isolation were monitored by phosphorus content (Chen et al., 1956), Folin protein determinations (lowry et al., 1951), dry weight determinations, phase contrast and polarized light microscopy, and interfacial properties. Surface activity was determined by bubble stability and clicking (Pattle, 1965), bubble resistance to antifoam, and behavior upon 0.15M NaCl in a modified Wilhelmy balance with a cycling time of 140 seconds (Greenfield and Kimmel, 1967).

The tracheal washes were centrifuged at 600 xg for 15 minutes to sediment cellular debris. The turbid supernatant liquid was recentrifuged at 16,000 xg for one hour, depositing an off-white pellet free of cellular material. After this second centrifugation 98% of the protein remained in the supernatant solution while 79% of the phosphorus sedimented. About 15 mg of this pelleted crude surfactant was consistently required to reduce surface tension to 6-7 dynes/cm upon compression of the air-saline interface. In saline the pellet produced a flocculant suspension which was dispersed by dialysis against 0.02M Tris-EDTA at pH 8.0, then against distilled water for 24 hours.

The deionized material was layered over a linear sucrose gradient of density 1.000-1.100 and centrifuged at $4^{\circ}C$ for 20 hours in a Spinco SW 39

rotor at 35,000 rpm. Three fractions were obtained: a white upper band, a white intermediate band centered at density 1.035, and a yellow pellet at the bottom of the tube (Fig. 1). Each fraction was dialyzed against

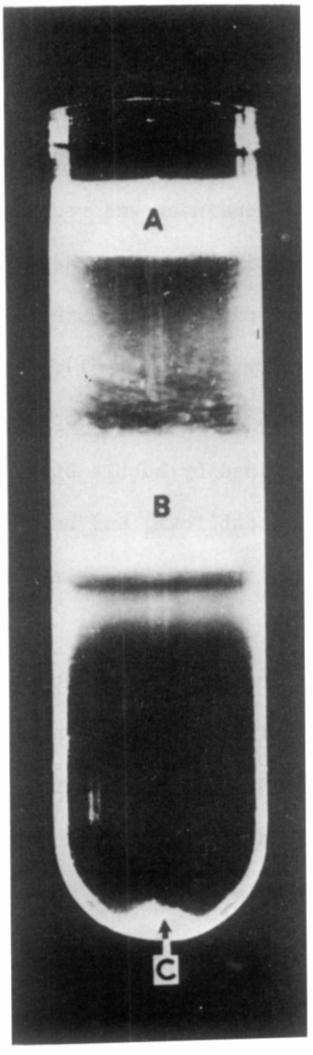

Fig. 1. Distribution of components obtained by equilibrium sucrose gradient centrifugation of the cell-free portion of tracheal washings. The intermediate band at B centered at density 1.035 contains the surfactant. For most animals, the small band below B was usually less prominent than shown here.

distilled water (the absence of sucrose was verified by thin layer chromatography), then lyophilized and resuspended in 0.15M NaCl for determining interfacial properties. Thirty-five µg of the intermediate band produced a large hystersis loop with minimum surface tension below 9 dynes/cm (Fig. 2), a bubble stability ratio of 0.94, clicking, and antifoam resistance. Because neither the upper band nor the lower pellet fulfilled any of these criteria, we concluded that the intermediate band contained the surfactant.

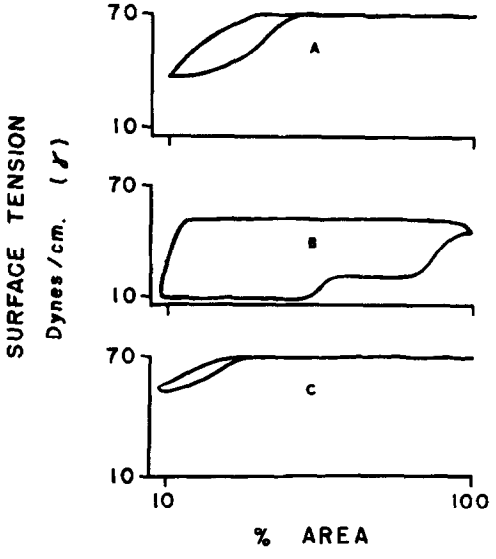

Fig. 2. Surface tension-area loops produced by layering the three fractions from the centrifuge tube shown in Fig. 1 upon 0.15M NaCl. A: floating band; B: intermediate band; C: mucoid pellet.

Based upon the results of the equilibrium gradient centrifugation, the isolation procedure was modified by layering crude surfactant over sucrose solutions of uniform density. The dialyzed pellet from the 16,000 xg centrifugation of the tracheal washings was layered over a sucrose solution of density 1.010 and centrifuged at 30,000 rpm in a Spinco 30 rotor for one hour. A pellet and a floating band were obtained. The floating material was discarded and the resuspended pellet was layered on a sucrose solution of density 1.055 and re-centrifuged for one hour at 40,000 rpm

in a Spinco 50 rotor. The surfactant floated while a yellow mucoid material sedimented. Additional mucoid material was removed by alternately sedimenting the surfactant in distilled water then floating it upon a sucrose solution of density 1.055. At each step a small yellow gelatinous pellet remained at the bottom of the tube. This procedure was repeated until no yellow pellet spun down. After dialysis the product fulfilled all the criteria of lung surfactant mentioned earlier.

The procedure described was carried out many times with consistent results, except in a few cases when the dogs showed signs of respiratory infection. In a representative isolation the yield was 0.76 mg of surfactant per gram wet weight of lung, or 14% of the dry weight of the dialyzed crude tracheal wash. Of the non-dialyzable phosphorus in the crude wash, 31% was recovered as surfactant. The phosphorus content was 28 µg/mg dry weight, a 2200-fold enhancement over that of the dialyzed crude wash.

CHEMICAL AND PHYSICAL CHARACTERIZATION

After lyophilization and desiccation over P_2O_5 in vacuo, the snow-white surfactant contained less than 0.5% by weight of material insoluble in 2:1 chloroform-methanol, but contained about 2% Folin protein. This proteolipid protein was denaturated by repeated evaporation of a chloroform-methanol-water emulsion of surfactant at 50°C in a rotatory evaporator, then removed by filtration. The filtrate contained less than 0.5% protein but satisfied all the established criteria for surfactant. Nine µg produced a large hysteresis loop with a minimum surface tension of 7 dynes/cm. For determination of carbohydrate, surfactant was hydrolyzed overnight at 100°C in 1N HCl then extracted with diethyl ether. Hexose was determined in the aqueous phase (Dische, 1949), and amounted to 0.7% of the dry weight of surfactant, expressed as glucose. The lipids in the surfactant were separated and identified by thin layer chromatography and compared with standards. The major lipids were phosphatidyl choline, phosphatidyl ethanolamine, cholesterol, and triglycerides. Smaller amounts of sphingomyelin, lysolecithin, free

fatty acids, and trace amounts of an identified spot with chromatographic properties similar to cardiolipin were detected. As reported by others, the fatty acid methyl esters prepared from the lecithin fraction were dominated by palmitate (60-70%). Suspended in water, lung surfactant appears as micelloids about 0.5 to 3.0 microns in diameter which are birefringent under polarized light microscopy. In the electron microscope, negatively stained preparations show the lamellar phase characteristic of phospholipids (Bangham and Horne, 1964). In saline a flocculant suspension is obtained.

DISCUSSION

Lung surfactant is best defined operationally, in terms of its properties. The material obtained, although essentially free of protein and carbohydrate, exhibits the physical properties of surfactant. These include a large hysteresis loop with minimum surface tension less than 10 dynes/cm, bubble clicking, stable bubbles, and antifoam resistance. Our isolation was carried out in aqueous media by monitoring activity, much as other biologically active materials are isolated. Surface-active lipids have been isolated in other laboratories by organic solvent extraction of centrifuged washings or whole lung homogenates. We wish to emphasize that our method does not employ drastic conditions which denature proteins. Organic solvents were used only to demonstrate that the 3% protein associated with the material is not essential for activity. Since the physical properties are unaltered by removal of protein, it is reasonable that the non-lipid fraction is a contaminant, perhaps mechanically entrained within the myelin forms of lipid. We conclude that the native surfactant does not contain functionally significant amounts of either protein or carbohydrate (Watkins, 1968). It is possible that the properties could be modified in the alveoli, where the liquid phase contains protein, mucopolysaccharides, and salts, but unlike serum lipoproteins lung surfactant is not a true lipoprotein.

The lipid analysis and minute protein content of lung surfactant sug-

gests that it is not of mitochondrial origin (Klaus et al., 1962). Mitochondria contain large amounts of cardiolipin and only minor amounts of cholesterol and triglycerides (Fleischer and Rouser, 1965). In mitochondria lipids exist in association with membrane proteins, and cannot be freed of protein by extraction with aqueous solvents.

SUMMARY

Lung surfactant was isolated from canine tracheal washings by differential centrifugation, equilibrium gradient centrifugation, and flotation on sucrose solutions. The preparation exhibits all the usual physical properties of surfactant, including a large hysteresis loop with low minimum surface tension, bubble clicking, bubble stability, and resistance to antifoam. It is a mixture of lipids, not a lipoprotein or a lipopolysaccharide. The total protein and carbohydrate content is less than 3%, and removal of these minor amounts of non-lipid materials does not affect surface properties. Phosphatidyl choline, phosphatidyl ethanolamine, cholesterol, and triglycerides are the major components. Other workers have extracted surface-active phospholipids from tracheal washings and whole lung homogenates by using organic solvents, which would denature lipoproteins. Since our isolation does not employ such drastic techniques but yields a product which is essentially free of protein and carbohydrate but retains the properties of surfactant, we conclude that native surfactant is a mixture of lipids.

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