Phospholipid and protein analysis of pulmonary surfactant

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Quantitative analysis of the phospholipid composition of pulmonary surfactant preparations has been achieved by a modification of the phosphorus-31 nuclear magnetic resonance method of E London and G Feigenson (J. Lipid Res. 20, 408-412, 1979).

Resolution of the protein components by a 2-dimensional isoelectric-focussing - SDS/ polyacrylamide-gel-electrophoresis technique is reported for the first time.

Two recently developed methods have been adapted for use in the analysis of pulmonary surfactant phospholipid and protein composition in order to monitor changes resulting from toxicological processes. A reliable and convenient phospholipid analysis method has been derived from the phosphorus-31 nuclear-magnetic-resonance approach of London and Feigenson (1979) by the use of a novel solvent system which improves sample solubility without sacrificing resonance sharpness or resolution. In addition a high-resolution protein separation has been achieved without sample delipidation and the concomitant loss of hydrophobic proteins both for lung-homogenate surfactant preparations and the more intractable material derived from lavage fluid.

A need for improved methods of phospholipid and protein analysis of surfactant was evident from examination of the available literature. Phospholipid estimation has usually been achieved by thin-layer-chromatographic (t.l.c.) methods although recently high-performance liquid chromatography has been applied, with limited success, to other biological samples. The t.l.c. methods have proved both time-consuming and unreliable, quantitation of phosphatidyl glycerol being particularly suspect. Reports of the phospholipid composition of surfactant preparations have consequently varied widely (Gil & Reiss, 1973; Einolf & Fenselau, 1974; King, 1974; Clements & King, 1976; Katyal et al. 1977; Baritussio et al., 1981; Slomiany et al., 1982, Young et al., 1982). Not all of this variation may be adequately attributed to differences in source and preparation methods.

Similar deficiencies were apparent in the methods available for surfactant protein estimation. No successful separation by electrophoretic techniques has been reported which reflects the complexity of the protein mixture suggested by detailed column analysis (Phizackerley et al., 1979). Most attempts at electrophoretic
separation have resulted in unsatisfactory resolution of the proteins and spreading of the bands (Clements & King, 1976; King et al., 1977; Katyal & Singh, 1979, 1981). In addition sample delipidation may have resulted in substantial losses of hydrophobic proteins (Phizackerley et al., 1979). Immunochemical purification (Sueishi and Benson, 1981) similarly may select a fraction of the total protein population.

To our knowledge the methods reported here represent new applications of the techniques described and offer significant advantages over other approaches reported to date.

Materials and Methods

Surfactant was prepared from hamster whole-lung homogenate or lavage fluid as described by Gratwohl et al. (1979).

Phospholipid determination

Surfactant phospholipids were extracted into chloroform:methanol as described by Einolf and Fenselau (1974) and dried under a stream of nitrogen. Samples (approx. 60 µg of phospholipid phosphorus) were dissolved in 0.3 ml of n-propanol; deuterium oxide:water (65:25:10 v/v) containing Triton X-100 (2.5%), disodium ethylenediaminetetraacetate (100 mM) and trizma base (100 mM, Sigma London Chemical Co.) at a nominal pH of 8.0 (glass electrode). Phosphorus-31 nuclear magnetic resonance (n.m.r.) spectra were obtained on a Jeol FX 90Q high-resolution fourier transform spectrometer operating a 60° pulse repeated every 2.2 s. Protons were continuously noise decoupled. 4K data points were collected and transformed with exponential broadening corresponding to 0.4 Hz. Chemical shifts were referred to an external 85% orthophosphoric acid solution. Samples were contained in 5-mm tubes at 'ambient' temperature (approx 35°C). Quantitation and chemical shifts were obtained by reference to suitable standard mixtures. No significant Nuclear Overhauser Enhancement effects were observed.

Protein electrophoresis

Surfactant preparations were concentrated by ultracentrifugation to produce a pellet of material. Concentration by lyophilization was found to render the samples insoluble. Solubilized protein specimens were obtained by adding 1 µl per µg protein of a solution containing urea (10 M), Triton X-100 (2%), NP-40 (2%), dithiothreitol (1%), carrier ampholines pH 3.5-10 (1%), ampholines pH 6-8 (0.5%) (L.K.B., Croydon, U.K.). Following brief sonicatation the samples were saturated with urea and allowed to stand at room temperature for a minimum of 1 h. Alizarin-red solution was then added (0.5 mg per ml in 50% ethanol) and the pH adjusted to approx. 3.5 with hydrochloric acid (4 M). Samples (100 µg of protein) were then submitted to isoelectric focussing (acid end application) and SDS/polyacrylamide-gel electrophoresis (7-16% acrylamide linear gradient) essentially as described by O'Farrell (1975). When the samples were solubilized in the manner described and applied to the acid end of the isoelectric focussing gels, the phospholipids did not appear to enter the gels.
during focussing. Alternatively whole-lung-homogenate surfactant samples (but not samples from lavage fluid) may be solubilized in SDS as described by Anderson and Anderson (1977) and applied to the basic end of the gels. Under these conditions phospholipid appeared to enter the gel and effected the focussing of proteins over part of the pH range. This was seen as horizontal streaking in part of the final 2-dimensional gel.

Proteins were detected by the silver staining method of Oakley et al. (1980). Coomassie blue was found to be ineffective.

One-dimensional separations were achieved by diluting the solubilized surfactant samples with the SDS solution of Anderson and Anderson (1977) and applying into a well in the Agar used to fix the isoelectric-focussing gel to the SDS gel. Applying into a well in the acrylamide stacking gel resulted in precipitation at the top of the gradient gel.

Results

Phospholipid analysis

Table 1 lists the chemical shifts of a number of synthetic phospholipids as observed in aqueous systems and in the n-propanol:water system described above. The phospholipid classes found in surfactant samples were well resolved from other phospholipids. Solubility of

<table>
<thead>
<tr>
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<th>Reported$^{abc}$</th>
<th>Aqueous$^{cd}$</th>
<th>Propanol$^c$</th>
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<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>(PC)$^e$</td>
<td>$^{+}0.90$</td>
<td>$^{-}0.88$</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>(PI)</td>
<td>$^{+}0.40$</td>
<td>$^{-}0.56$</td>
</tr>
<tr>
<td>N,N dimethylphosphatidyldimethanolamine</td>
<td>$-$</td>
<td>$-$</td>
<td>$^{-}0.56$</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>(LPC)</td>
<td>$^{+}0.15$</td>
<td>$^{+}0.38$</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>(PS)</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>(PE)</td>
<td>$^{+}0.25$</td>
<td>$^{+}0.20$</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>(SM)</td>
<td>$^{\pm}0.00$</td>
<td>$^{-}0.20$</td>
</tr>
<tr>
<td>Diphosphatidylglycerol</td>
<td>(DIPG)</td>
<td>$^{-}0.31$</td>
<td>$^{+}0.26$</td>
</tr>
<tr>
<td>Lysophosphatidyl-</td>
<td></td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>ethanolamine</td>
<td>(LPE)</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
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<td>$^{-}0.43$</td>
<td>$^{+}0.38$</td>
</tr>
<tr>
<td>Lysophosphatidylglycerol</td>
<td>(LPG)</td>
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<td>$-$</td>
</tr>
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$^b$Note opposite sign convention.
$^c$p.p.m. relative to phosphoric acid.
$^d$Triton-X-100-containing system pH 8.0.
$^e$Abbreviations are given in parentheses.
surfactant phospholipid was poor in the aqueous systems but good in
the propanol-containing system (15-mg-per-ml solutions were achieved
without difficulty). The quality of spectra obtained with surfactant
samples is illustrated in Fig. 1. The broadening of the resonance
signals frequently observed in organic solvents (London & Feigenson,
1979) was not seen in this mixed solvent. In addition this figure
illustrates the quantitation possible by measuring peak areas. The
standard mixture was made up by weight to the composition indicated
by analysis of the whole-lung surfactant sample. On analysis the
standard mixture gave the expected peak areas to within 1-2%. With
a sample of 60 μg phospholipid phosphorus components representing 1%
of the mixture could be readily detected.

Protein electrophoresis

An example of the quality of separation obtainable for hamster
whole-lung surfactant samples is given in Fig 2. Proteins which
appear to be of plasma origin were detected by comparison with

![Fig. 1. Phosphorus-31 n.m.r. of surfactant lipids and synthetic standard mixture. Abbreviations as in Table 1.](image-url)
Fig. 2. 2-dimensional isoelectric focussing/SDS PAGE of surfactant prepared from lung homogenate. Major surfactant proteins are shown arrowed. To the right of the main gel a 1-dimensional separation of the surfactant sample is shown. To the left are molecular-weight markers. Isoelectric-point calibration was made by reference to known standards which were run in other gels under identical conditions.

samples of plasma treated similarly. Although there were a limited number of major proteins associated with surfactant a considerable number of other minor proteins of non-plasma origin were seen.

Discussion

The technique described here for phospholipid analysis of surfactant preparations compares favourably with thin-layer chromatographic methods both for cost and precision. A clear disadvantage of the n.m.r. approach is that it is not a separation method so that radio-tracer work, for example, will continue to require a chromatographic method. None the less the technique reported here provides a valuable routine method for analysis of surfactant samples and should be directly applicable to other sources of phospholipid mixtures.

An adequate electrophoretic separation of the protein components of surfactant (which constitute approx. 10% of the material present) has been complicated by the interference of the large proportion of phospholipid present. The technique described here appears to provide a solution to this problem without sacrificing the integrity of the sample by applying preparation methods which may lead to selective
losses of certain proteins. Comparison of the 2-dimensional patterns with 1-dimensional separations suggests that all but a few minor high-molecular-weight proteins are visualized on the 2-dimensional separation. Those lost presumably have isoelectric points outside the range (3.5-10.0) studied, since there is no evidence of protein precipitation either at the point of application to the isoelectric focussing gel or at the top of the SDS gel.

The methods should be of considerable utility in allowing the study of factors which affect surfactant composition and in aiding the identification of minor protein components which may possess physiological significance in surfactant function.

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References