A New Technique for Studying the Relationship between Maternal Diabetes and the Sialic Acid Content of Fetal Pulmonary Surfactant

Thomas Delahunty

By adapting a standard method for precipitation of high-density lipoprotein cholesterol with phosphotungstic acid (PTA) and Mg2+, fetal pulmonary surfactant can be rapidly isolated from human amniotic fluid, 97% of the total disaturated phosphatidylcholine being precipitated from the sample. The lecithin/sphingomyelin ratio for 17 separate specimens correlated reasonably well (r = 0.76) with the concentration of disaturated phosphatidylcholine in the PTA precipitate. Using thiobarbituric acid as the chromophore, I measured sialic acid in the PTA precipitate after overnight treatment with neuraminidase. The sialic acid/protein ratio for the PTA precipitate was identical to that for the surfactant, as isolated by ultracentrifugation. The concentrations of insulin and C-peptide were significantly greater in specimens of amniotic fluid from mothers with diabetes than from non-diabetic mothers (p < 0.001). When the specimens were segregated according to a C-peptide cutoff value of 4 μg/L, there was a small, significant decrease in PTA-precipitated concentrations of sialic acid in the samples with C-peptide > 4 μg/L. The results suggest a possible mechanism for the increased incidence of respiratory distress among infants born to diabetic mothers.

Additional Keyphrases: pregnancy • fetal status • respiratory distress syndrome • amniotic fluid • lecithin/sphingomyelin ratio • phosphatidylcholine • insulin • C-peptide • neuraminic acid

The increased prevalence of respiratory distress syndrome among the neonates of diabetic women is probably not related to an inability of the granular pneumonocytes to synthesize the surface active dipalmitoyl phosphatidylcholine (2, 3). However, the possibility that delivery of this lipid to the air-water interface is somehow affected by apparent hyperinsulinemia in the neonatal circulation (4, 5) is intriguing. Because the hyperinsulinemia or hyperglycemia (or both) apparently stimulate the formation of glycoproteins in the pneumonocytes (3, 6), this physiological effect might be mediated by a derangement in carbohydrate metabolism. In this regard, sialic acid (neuraminic acid) is an interesting monosaccharide to measure: its metabolism is related to that of glycogen (7) and it is covalently bound to surfactant apoprotein (8). Thus, one of the aims of this study was to investigate the possibility that fetal hyperinsulinemia might decrease the apoprotein-bound sialic acid within the surfactant–lipoprotein complex. The apparent inverse correlation between concentrations of surfactant apoprotein in amniotic fluid and the subsequent development of respiratory distress syndrome in infants of diabetic mothers (9) emphasizes the importance of the apoprotein in surfactant function.

Furthermore, because previous reports of concentrations of insulin and C-peptide in amniotic fluid have not been consistently correlated with maternal diabetic status (5, 10, 11), another aim was to establish the usefulness of C-peptide in amniotic fluid as a means of monitoring maternal diabetic control and fetal production of endogenous insulin. To conveniently measure apoprotein sialic acid concentrations in apoprotein, I adapted a simple technique routinely used for plasma lipoproteins (12, 13) to rapidly precipitate surfactant from amniotic fluid.

Materials and Methods

Specimens of amniotic fluid from women at 35 to 41 weeks of gestation were promptly centrifuged at 800 × g for 15 min at 4°C, and the supernates were stored at −10°C until analysis. The obstetrician's assessment of the diabetic status of the patients was based on results for plasma glucose and glucose tolerance tests as well as on the degree of dependence on exogenous insulin. All classes of diabetics except those with gestational diabetes were included in this study.

The lecithin/sphingomyelin ratio (L/S) in these supernates was determined routinely according to Kulovich et al. (14). The concentrations of insulin and C-peptide in amniotic fluid were determined with Pharmacia ("Phadeseph") and Mallinckrodt ("RIA-Quant") RIA kits, respectively. The sensitivity (limit of detection) of each method was <2.5 milli-int. units/L and <0.3 μg/L, respectively, and the respective within-run CVs were 12.8% and 15% (n = 20 each).

Surfactant was isolated from amniotic fluid by phosphotungstic acid (PTA) precipitation (HDL precipitation reagent; Sigma Chemical Co., St. Louis, MO) as follows: Incubate 1 mL of amniotic fluid at 37°C for 30 min in the presence of 0.2 mL of PTA reagent (final concentrations of PTA and Mg2+, 1.6 and 67 mmol/L, respectively). Centrifuge at 800 × g for 15 min and re-suspend the precipitate in 1 mL of PTA reagent diluted fivefold with isotonic saline. Repeat the centrifugation step, then analyze the washed PTA precipitate, as described below.

Surfactant was also isolated from amniotic fluid by conventional ultracentrifugation at a density of 1.1 kg/L as previously described (15).

Sialic acid in the PTA precipitate was determined by incubation for 16 h at 37°C with 100 μL of neuraminidase (EC 3.2.1.18; Sigma Chemical Co.), 1 g/L, in acetate buffer (10 mmol/L, pH 5.0). A prior study involving incubations at 3, 12, 16, and 40 h showed that the release of free sialic acid in this system did not increase after about 12 h; evidently the 16-h treatment with the enzyme sufficed for maximum hydrolysis. I then estimated free sialic acid, using one-fifth of the volumes of samples and reagents recommended by Roboz et al. (16). The within-run CV so obtained was 22% at a sialic acid concentration of 2.9 μmol/L (n = 20).

A pilot study with 12 aliquots of pooled amniotic fluid was performed to determine whether unreacting O-acetylated sialic acid was being released by the treatment with neuraminidase. Six of the 1-mL samples were subjected to mild acid hydrolysis (17), six left untreated after enzymic hydroly-
ysis. There was no significant increase in the absorbance values obtained, suggesting that O-acetylation is not a major factor in the determination.

I measured the protein in the PTA precipitate with a kit for protein assay (Bio-Rad, Richmond, CA 94804) based on the method of Bradford (18). I calculated the sialic acid/protein molar ratio by using 69,000 Da for the molecular mass of the protein (19).

Disaturated phosphatidylcholine in the amniotic fluid and in the precipitates with PTA was determined by the method of Mason et al. (20), previous experiments having demonstrated the usefulness of this technique for quantifying this analyte in preparations of pulmonary surfactant (21, 22).

Results

To assess the validity of using the phosphotungstic acid procedure for isolating surfactant from amniotic fluid, I compared the disaturated phosphatidylcholine content of the PTA precipitate with that of the supernate. For 19 specimens from different women, disaturated phosphatidylcholine in the precipitate accounted for 96.8% (SD = 6.6%) of the total disaturated phosphatidylcholine in the sample. Moreover, the L/S ratio for the supernate correlated well (r = 0.76) with the disaturated phosphatidylcholine in the PTA precipitate for 17 separate specimens. These data support the concept that in the PTA method most of the surfactant was isolated from amniotic fluid.

The sialic acid released by neuraminidase treatment from the PTA precipitate was measured and compared with the amount of protein in that fraction for 12 separate specimens of amniotic fluid. The molar ratio obtained [2.07 (SD = 0.6), sialic acid/protein] differed insignificantly (p > 0.05) from that obtained by ultracentrifugation [1.97 (SD = 0.6), sialic acid/protein], which suggests that measurement of sialic acid release from PTA precipitates provides a reliable estimate of the content of that monosaccharide in the fetal surfactant apoprotein.

Table 1 shows the concentrations of insulin and C-peptide in amniotic fluid specimens from diabetic and non-diabetic women. Insulin and C-peptide both differed significantly between diabetics and non-diabetics, and there was a reasonably good correlation between insulin and C-peptide for specimens from diabetic and non-diabetic patients. However, C-peptide concentration and the L/S ratio were not correlated in either diabetic or non-diabetic patients.

Because the amniotic-fluid specimens were from patients with various degrees of diabetic control, I separated them on the basis of C-peptide concentrations, using a cutoff value of 4 μg/L. As Table 2 shows, there was no difference in the L/S ratio between these two groups of patients, but there was a small but significant difference in the PTA-precipitated sialic acid (p < 0.04). Furthermore, the L/S ratio was not correlated with the concentrations of PTA-precipitated sialic acid.

### Table 1. Comparison of Components of Amniotic Fluid from Diabetic and Non-Diabetic Women

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>C-peptide</th>
<th>r</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD, mili-int. units/L</td>
<td>Mean ± SD, μg/LD</td>
<td>Insulin vs L/S</td>
</tr>
<tr>
<td>Diabetics</td>
<td>12</td>
<td>20.7 ± 16</td>
<td>14</td>
</tr>
<tr>
<td>Nondiabetics</td>
<td>27</td>
<td>5.6 ± 8*</td>
<td>33</td>
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</tbody>
</table>

*Significantly different (p < 0.001) from diabetic.

### Table 2. Comparison of L/S and PTA-Precipitated Sialic Acid in Various Amniotic Fluid Specimens

<table>
<thead>
<tr>
<th></th>
<th>L/S</th>
<th>C-peptide</th>
<th>n</th>
<th>μmol/L</th>
<th>n</th>
<th>r</th>
</tr>
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<tbody>
<tr>
<td>C-peptide &gt; 4 μg/L</td>
<td>3.35 ± 0.95</td>
<td>10</td>
<td>2.95 ± 0.74</td>
<td>11</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>C-peptide &lt; 4 μg/L</td>
<td>3.20 ± 1.80</td>
<td>43</td>
<td>3.70 ± 1.10*</td>
<td>43</td>
<td>0.11</td>
<td></td>
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*Significantly different (p < 0.04) from results with C-peptide >4 μg/L. Results expressed as the mean ± SD.

Discussion

The routine use of polyanionic precipitation as a means of separating low-density lipoprotein from serum was first proposed by Burstein et al. (23). Since then, sulfated polysaccharides such as heparin have been used most commonly (12, 24), but PTA has become increasing popular; it is relatively inexpensive, causes fewer problems with enzymic analysis, and leads to analytical recovery of phosphatidylcholine that is almost identical with that for ultracentrifugation (13). The results described here support the concept that the PTA precipitation technique will also quantitatively isolate disaturated phosphatidylcholine from amniotic fluid—a hypothesis further supported by the observed good correlation between the L/S ratio of disaturated phosphatidylcholine in the PTA precipitate and the L/S ratio of disaturated phosphatidylcholine in the amniotic fluid.

The possibility that measurement of phospholipid in the PTA precipitate represents a new and more convenient method for detecting fetal lung maturity, as compared with the L/S ratio, remains to be more extensively assessed by retrospectively comparing neonates with and without respiratory distress. Because I was interested in measuring the sialic acid in the surfactant apoprotein, the finding that the sialic acid/protein ratio in the PTA precipitate was identical to that obtained in the surfactant prepared by a conventional technique indicated that I could use the precipitation method for this study.

The finding that the concentrations of insulin and C-peptide in amniotic fluid were both highly significantly increased in maternal diabetes differs somewhat from the results reported by Lin et al. (5), which indicated that C-peptide differs more highly significantly from normal than does insulin. On the other hand, Tchobroutsky et al. (10) found insulin to be superior to C-peptide in discriminating between diabetics and non-diabetics, and Stangenberg et al. (11) could find no significant difference when C-peptide alone was measured. These apparently discordant results are probably best explained by the different RIA techniques used and by the criteria used in selecting the diabetic patients.

When I compared the insulin and C-peptide values, the resulting correlation was similar to that of Tchobroutsky et al. (10) for non-diabetic patients but, unlike them, I could find no difference in correlation between the diabetic and non-diabetic patients. The findings that the L/S values were indistinguishable between fluids grouped according to C-peptide concentrations, and the lack of correlation between these two variables, as reported here, are in agreement with the results of Lin et al. (5, 25). Thus my results support the view that either insulin or C-peptide concentrations in amniotic fluid can be measured to assess the influence of maternal diabetes on the endogenous production of fetal insulin, although measurement of these peptides alone cannot be substituted for the L/S ratio in assessing the status of the fetal lungs.

Since the rationale for measuring sialic acid concentra-
tions in surfactant apoprotein was to test the hypothesis that fetal hyperinsulinemia was somehow inhibiting the biosynthesis of that monosaccharide, the finding that the values were significantly decreased for amniotic fluids containing increased C-peptide concentrations supported this concept. Moreover, the negative correlation between surfac-
tant apoprotein and the incidence of respiratory distress syndrome reported by Katyal et al. (9) could also be ascribed to a deficiency in sialic acid, because their ELISA technique may be detecting antigenic sialic acid residues on the apoprotein (26).

Because synthesis of disaturated phosphatidylcholine is apparently unaffected in the fetal pneumocytes of diabetic animals (2), the high incidence of respiratory distress syndrome in this condition (1) could instead be due to a deficiency in apoprotein or carbohydrate content of surfac-
tant. Considering that protein-bound sialic acid has been implicated as a regulatory factor in the uptake of macromolecules by hepatocytes (27) and in cellular adhesion (28), one can postulate that the negatively charged sialic acid moiety assists the surfactant apoprotein in its role at the air–water interface (29). Therefore, a subtle decrease in the apoprotein content of sialic acid, as reported here, might be expected to have a negative impact on neonatal respiration. Further studies with fluids from mothers whose infants subsequently developed respiratory distress syndrome are planned, to investigate this postulate.

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