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# THE MEASUREMENT OF PHOSPHATIDATE PHOSPHOHYDROLASE IN HUMAN AMNIOTIC FLUID

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## Summary

Phosphatidate phosphohydrolase (EC 3.1.3.4) activity can be found in late gestational human amniotic fluid and is thought to originate in type II alveolar cells of the fetal lungs where it plays an important role in lung surfactant synthesis. In the present study, phosphatidate phosphohydrolase activity was detected and characterized in a 105 000 $\times g$ pellet of amniotic fluid using either [$^{32}$P]phosphatidate or a water-soluble analog, 1-$O$-hexadecyl-$rac$-[2-$^3$H]glycerol 3-phosphate as substrate. With either substrate, enzyme activity was optimal at pH 6.0. The soluble analog was hydrolyzed with a $K_m$ value of 163 $\mu$M and a $V$ of 30 nmol/min per mg of protein, and offered several advantages over phosphatidate as a substrate for assaying phosphatidate phosphohydrolase in amniotic fluid. Using the synthetic analog, phosphatidate phosphohydrolase activity was measured in the 700 $\times g$ supernatant fraction of 30 human amniocentesis samples and compared with another index of fetal lung maturity, the phosphatidylcholine/sphingomyelin ratio. The results suggest that the new phosphohydrolase assay may be clinically useful in the assessment of fetal lung development.

## Introduction

Because of its role in facilitation of gas transport, the lung alveolus is inherently a delicate and unstable structure. The tendency to collapse is counteracted by a lipoprotein surfactant which reduces alveolar surface tension. Dipalmitoyl phosphatidylcholine is the phospholipid present in greatest amounts in the pulmonary surfactant and is believed to be largely responsible for the observed surface-active properties [1]. During fetal lung development, the surfactant is found in the amniotic fluid in increasing

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Abbreviation: 1-alkylglycerol-3-$P$, 1-$O$-hexadecyl-$rac$-glycerol 3-phosphate

amounts and its measurement has been used to assess fetal lung maturity in the prevention of respiratory distress syndrome of the newborn [2—5]. In pregnancies in which the extent of fetal lung development must be determined, measurement of the phosphatidylcholine/sphingomyelin ratio in amniotic fluid has become an accepted procedure [2].

The biosynthesis of pulmonary surfactant phosphatidylcholine has received considerable attention, and in most species the major pathway appears to proceed via CDPcholine [6]. Several studies have sought to identify an enzyme regulating dipalmitoyl phosphatidylcholine synthesis in fetal lung. The activities of most of the enzymes in the biosynthetic pathway either remain largely unchanged throughout gestation, or else change in a manner difficult to relate to the observed prenatal surge in surfactant phosphatidylcholine synthesis [7—11]. However, the activities of lysophosphatidylcholine : lysophosphatidylcholine acyltransferase in mouse lung and phosphatidate phosphohydrolase in rabbit lung have been shown to increase dramatically prior to the detectable initiation of surfactant synthesis [12,13]. Furthermore, phosphatidate phosphohydrolase appears to be secreted along with the surfactant and its presence can be detected in human amniotic fluid [14]. This enzyme has been extensively studied in other tissues. In liver, there is evidence that it may be the rate-limiting enzyme in the synthesis of both neutral lipid [15] and glycerophospholipid [16]. It is not unreasonable therefore to suspect that phosphatidate phosphohydrolase plays a role in regulating surfactant phosphatidylcholine synthesis.

Johnston and co-workers [14] have described the relationship between levels of phosphatidate phosphohydrolase and the phosphatidylcholine/sphingomyelin ratio in amniotic fluid and have suggested that measurement of amniotic phosphatidate phosphohydrolase may be useful for evaluating fetal lung maturation [14]. However, despite the considerable interest in phosphatidate phosphohydrolase, there has not been a convenient, sensitive assay for this enzyme. In a recent study of phosphatidate phosphohydrolase in guinea-pig brain [17] we found lysophosphatidate (1-acylglycerol 3-phosphate) to be a good substrate for the enzyme and a sensitive assay was developed utilizing 1-alkyl-[2-$^3$H]-glycerol 3-phosphate as substrate [8]. In the present study we describe the application of this assay to human amniotic fluid phosphatidate phosphohydrolase and examine its usefulness as a possible clinical assay for the enzyme.

## Materials and Methods

Samples of amniotic fluid were taken by amniocentesis for routine clinical analysis and portions remaining were kindly made available by Dr. K.M.J. Menon, Department of Obstetrics and Gynecology, University of Michigan. To remove contamination by intact cells and debris, amniotic fluid samples were centrifuged at $700 \times g$ for 5 min at $4°C$ and the pellet was discarded. In experiments in which the relationship between phosphatidate phosphohydrolase activity and the phosphatidylcholine/sphingomyelin ratio was investigated, the $700 \times g$ supernatant was used directly. When characteristics of the enzyme in amniotic fluid were investigated (see Figs. 1, 2 and 3), a further centrifugation at $105\,000 \times g$ for 60 min was performed [13]. Samples of amniotic fluid (6—8

of gestational age 34—38 weeks) were routinely pooled. The combined sample was centrifuged at 105 000 $\times g$ for 60 min. The pellet was resuspended in water using a glass-glass homogenizer.

Acid phosphatase (potato B grade) was obtained from Calbiochem, Los Angeles, Calif. Alkaline phosphatase (calf intestine Type VII), chimyl alcohol, hexadecyl iodide, $p$-nitrophenylphosphate and tris(hydroxymethyl)amino-methane (Trizma Base) were from Sigma Chemical Co., St. Louis, Mo. 2-Amino-2-methyl-propan-1-ol was supplied by Eastman Organic Chemicals, Rochester, N.Y. Bovine serum albumin Pentex®, was purchased from Miles Labs., Inc., Elkhart, Ind. Sodium boro [³H]hydride (NaB³H₄, 8 Ci/mmol) was supplied by Amersham/Searle Corp., Arlington Heights, Ill. Other chemicals were of commercially available reagent grade.

*Preparation of 1-O-hexadecyl-rac-[2-³H]glycerol 3-phosphate.* 1-O-Hexadecyl dihydroxyacetone 3-phosphate was synthesized as outlined by Hajra [19], except that the intermediate 2-O-hexadecyl glycolic acid was synthesized by refluxing hexadecyl iodide, ethyl glycolate and Ag₂O in a molar ratio of 1 : 2.5 : 1.5 in xylene for 15 h. 1-O-Hexadecyl-rac-[2-³H]glycerol 3-phosphate (1-alkylglycerol 3-$P$) was prepared from 1-O-hexadecyl dihydroxyacetone phosphate by reducing with NaB³H₄ [20]. The product was purified by column chromatography (Unisil, Clarkson Chemical Co., Inc., Williamsport, Pa). Non-polar contaminants were eluted with three column volumes of chloroform followed by five column volumes of chloroform/methanol (4 : 1, v/v) to elute 1-alkylglycerol-3-$P$. Purity of the substrate was assessed by thin-layer chromatography on silica gel G plates (Uniplate, Analtech Inc., Newark, Del.) developed in chloroform/methanol/acetic acid/water (100 : 40 : 12 : 4, v/v) followed by fluorography [21]. Specific radioactivity of the substrate was determined using the phosphate assay of Ames and Dubin [22] and measuring radioactivity in an ethanol/toluene scintillant (200 ml ethanol, 3640 ml toluene, 20 g PPO and 0.4 g POPOP). The purified substrate was stored in chloroform/methanol (2 : 1, v/v) at −20°C.

*Preparation of [³²P]phosphatidate.* The preparation of membrane-bound [³²P]phosphatidate is described elsewhere [17]. In preliminary experiments it was observed that phosphatidate in this form was not a good substrate for amniotic fluid phosphatidate phosphohydrolase. However, aqueous dispersions of [³²P]phosphatidate were much more rapidly hydrolyzed by the enzyme. Total lipid was extracted from [³²P]phosphatidate-enriched microsomes using an acidic extraction procedure [23]. Most of the radioactivity (>95%) was recovered in phosphatidate. Small amounts of the total lipid extract were mixed with purified unlabelled phosphatidate prepared from egg phosphatidyl-choline [24,25], and this mixture was used to prepare aqueous dispersions of substrate.

*Assay of phosphatidate phosphohydrolase.* (1) [³²P]Phosphatidate as substrate: In some experiments the characteristics of 1-alkylglycerol-3-$P$ and [³²P]phosphatidate hydrolysis by phosphatidate phosphohydrolase were com-pared. Aqueous dispersions of [³²P]phosphatidate were prepared by sonicating in 70 mM Tris/maleate buffer (pH 6.0) for 60 s (Bransonic 2, 60Hz-40W, Heat Systems-Ultrasonics Inc., Plainview, N.Y.). Assays were performed at 37°C for 60 min and contained 3.5 $\mu$mol of Tris/maleate buffer (pH 6.0), 0.54 $\mu$mol of

[$^{32}$P]phosphatidate (approx. 0.08 Ci/mol) and amniotic fluid $105\,000 \times g$ pellet (50—150 $\mu$g protein) in a total volume of 100 $\mu$l. Incubations were started by the addition of enzyme and terminated by mixing with 1.1 ml of chloroform/methanol (1 : 2, v/v). After adding 0.6 ml of 0.1 M HCl and 0.6 ml of chloroform, samples were again vortex-mixed and then centrifuged ($700 \times g$ for 5 min). An aliquot (0.8 ml) of the upper phase was added to 9 ml of Triton scintillant (780 ml of Triton X-100, 2100 ml of toluene, 15 g PPO and 0.3 g POPOP) and radioactivity determined.

(2) 1-O-Hexadecyl [2-$^3$H]glycerol 3-phosphate as substrate: Suspensions of 1-alkylglycerol-3-P (1 mM) were prepared by warming the lipid to 40°C in 70 mM Tris/maleate buffer (pH 6.0) and sonicating briefly (3 × 20 s). Incubations were at 37°C in a shaking water bath and contained in a final volume of 100 $\mu$l, 3.5 $\mu$mol Tris/maleate buffer (pH 6.0), 50 nmol 1-alkylglycerol-3-P (approx. 20 Ci/mol) and phosphatidate phosphohydrolase (5—100 $\mu$g protein). When the enzyme activity was measured in amniotic fluid-$700 \times g$ supernatant, EDTA and bovine serum albumin were also included in the assay medium at final concentrations of 5 mM and 5 mg/ml, respectively. Unless otherwise stated, incubations were started by the addition of substrate and were for 20 min. To terminate incubations, 1.4 ml ice-cold 20 mM NaOH and 2.0 ml ice-cold diethyl ether/ethanol (3 : 1, v/v) were added. After vortex-mixing (10 s) and centrifugation ($700 \times g$ for 1 min) a sample (1.0 ml) of the upper phase was dried in a scintillation vial and 10 ml ethanol/toluene scintillant were added.

*Other methods.* Acid phosphatase was assayed in sodium citrate buffer (100 mM, pH 5.6) at 37°C, using p-nitrophenylphosphate as substrate [26]. With the same substrate, alkaline phosphatase was determined at 25°C in 150 mM 2-amino-2-methyl-propan-1-ol buffer (pH 10.0)/1.5 mM MgCl$_2$ by recording the increase in absorbance at 405 nm [27]. Protein was determined by the method of Lowry et al. [28], with bovine serum albumin as standard. Phosphatidylcholine/sphingomyelin ratios were determined as described by Bryson et al. [29].

## Results

We have reported elsewhere the value of 1-alkylglycerol-3-P as a substrate for a purified preparation of the soluble form of brain phosphatidate phosphohydrolase [18]. When this assay was applied directly to either whole amniotic fluid or the $700 \times g$ supernatant, unexpectedly low phosphohydrolase levels were measured. In preliminary studies we found, as did Jimenez et al. [14], using phosphatidate as substrate, much higher levels of the phosphohydrolase in amniotic fluid by examining a $105\,000 \times g$ pellet. Therefore, we first established optimal assay conditions using a $105\,000 \times g$ pellet and then developed a suitable method for the low-speed supernatant fraction.

*Dependence of phosphatidate phosphohydrolase activity on pH*

Fig. 1 shows the effect of pH on phosphatidate phosphohydrolase in a $105\,000 \times g$ pellet using either aqueous dispersions of phosphatidate or 1-alkylglycerol-3-P as substrate. The observed optimum at pH 6.0 agrees with earlier

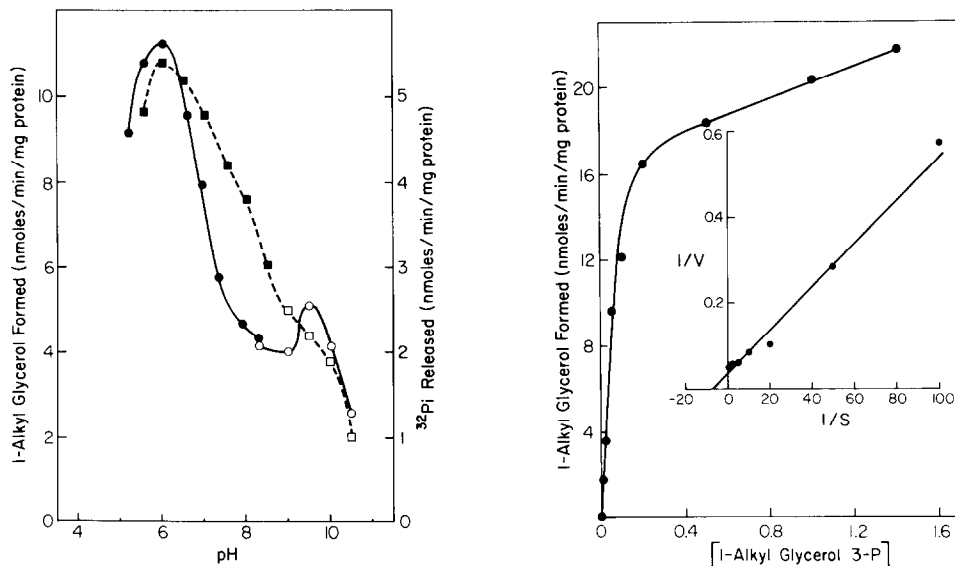Fig. 1. The effect of pH on phosphatidate phosphohydrolase activity. Enzyme activity was measured in the amniotic fluid-105 000 X $g$ pellet using either [$^{32}$P]phosphatidate (■,□), or 1-alkyl-[2-$^3$H]glycerol-3-$P$ (●,○) as substrate. Assay conditions were at 37°C for 20 min as described in the text. The buffers used were, 35 mM Tris/maleate (■,●, pH 5.2—8.3) and 40 mM 2-amino-2-methyl-propan-1-ol (□,○, pH 8.3—10.5). Values are means of three determinations.

Fig. 2. The dependence of phosphatidate phosphohydrolase activity on 1-alkylglycerol-3-$P$ concentration. The assays employed the 105 000 X $g$ pellet from six pooled amniotic fluid samples. Different substrate concentrations were achieved by adding varying amounts of 1.5 mM 1-alkyl-[2-$^3$H]glycerol-3-$P$ in 70 mM Tris/maleate buffer (pH 6.0). Other assay conditions were as described in the text. The data are from three experiments.

work [14]. A secondary peak of activity was observed at pH 9.5 when 1-alkyl-glycerol-3-$P$ was the substrate. This may represent activity of alkaline phosphatase which is known to be present in amniotic fluid [30]. Using a variety of criteria Jimenez et al. [14] were able to show that the hydrolysis of phosphatidate by amniotic fluid is not attributable to alkaline phosphatase. However, since 1-alkylglycerol-3-$P$ is much more water soluble than phosphatidate and because of the known broad specificity of alkaline phosphatase, the possible interference by alkaline phosphatase with the present assay for phosphatidate phosphohydrolase was examined (see below).

*Effect of 1-alkylglycerol-3-P concentration on phosphatidate phosphohydrolase*
   The dependence of phosphatidate phosphohydrolase activity on 1-alkyl-glycerol-3-$P$ concentration is shown in Fig. 2. A reciprocal plot of the data shown yields a $K_m$ value of 163 $\mu$M and a $V$ 30 nmol/min per mg protein. Similar experiments (not shown) using phosphatidate as substrate gave a $K_m$ value of approx. 4.5 mM. The continued rise in activity at concentrations of 1-alkylglycerol-3-$P$ above 1 mM may reflect the detergent properties of this substrate. Since phosphatidate phosphohydrolase in amniotic fluid is believed to be associated with lamellar bodies [13], high concentrations of 1-alkyl-glycerol-3-$P$ may cause release of sequestered enzyme from these structures.

Alternatively, the continued rise in activity may be the result of preference by the enzyme for the micellar form of the substrate analog. Routinely, the assay medium for the 105 000 × $g$ pellet contained 35 mM Tris/maleate buffer (pH 6.0) and 0.5 mM 1-alkylglycerol-3-$P$. The assay was linear with time for at least 60 min and with protein concentration up to 2 mg/ml.

## Heat denaturation of phosphatidate phosphohydrolase

Fig. 3 shows that the rate of disappearance of 1-alkylglycerol-3-$P$ phosphohydrolase activity closely parallels loss of phosphatidate phosphohydrolase activity during heat denaturation. This suggests that both substrates are hydrolyzed by the same enzyme(s). The data, plotted as log percent activity remaining against time at 70°C (see inset), indicate that the process is not first order, and suggest the presence of more than one enzyme or of different forms of the same enzyme. This heat stability was unexpected in view of studies of the same enzyme in other tissues [15,17]. It was suspected that this could be in some way related to the association of the amniotic fluid enzyme with lamellar bodies. However, when we prepared lamellar body-free microsomes from adult rat lung essentially as described for pig lung by Spitzer et al. [31], microsomal phosphatidate phosphohydrolase was also relatively heat stable (45% activity remaining after 15 min at 70°C). It is possible that there is a difference between lung phosphatidate phosphohydrolase and forms from other tissues.
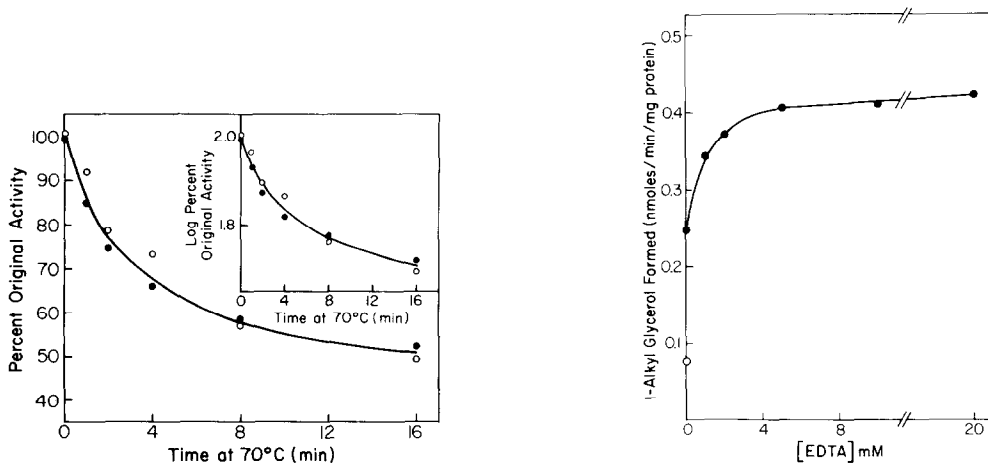


Fig. 3. The heat-denaturation of phosphatidate phosphohydrolase. Aliquots of amniotic fluid-105 000 × $g$ pellet were sealed in assay tubes and heated for different lengths of time at 70°C in a metal block. After heating, each tube was placed on ice for 5 min before centrifuging (700 × $g$ for 5 min). While there was no actual sedimentation of the preparation, heating did increase turbidity. Assays were started by the addition of either 1-alkyl-[2-$^3$H]glycerol-3-$P$ (●) or [$^{32}$P]phosphatidate (○) and were conducted as described in Materials and Methods. Mean of four determinations.

Fig. 4. The effect of EDTA on 1-alkylglycerol-3-$P$ hydrolysis by amniotic fluid-700 × $g$ supernatant. The data shown are the means of four experiments with 700 × $g$ supernatant from four pooled samples of amniotic fluid. Varying amounts of disodium EDTA in 70 mM Tris/maleate buffer (pH 6.0) were added to the assay medium described in the text, in the presence (●) of 5 mg bovine serum albumin/ml. ○, represents activity in the absence of both EDTA and albumin (see text).

*Measurement of phosphatidate phosphohydrolase in amniotic fluid-700 $\times g$ supernatant*

Table I, in agreement with earlier work [13], demonstrates that most of the phosphatidate phosphohydrolase activity of amniotic fluid is membrane bound. In preparing the 105 000 $\times g$ pellet from amniotic fluid-700 $\times g$ supernatant, the specific activity of phosphatidate phosphohydrolase (1-alkylglycerol-3-$P$ as substrate) was increased approx. 40-fold (Table I). However, recovery of activity at this step was about 215%, indicating activation (or release of inhibition) of the enzyme. The 105 000 $\times g$ supernatant was found to have very little phosphatidate phosphohydrolase activity, and when added back to the 105 000 $\times g$ pellet, it produced a pronounced inhibition. The same supernatant also inhibited purified phosphatidate phosphohydrolase from rat liver prepared as described by Lamb and Fallon [15]. The inhibitory factor was heat stable, dialyzable and could be accounted for by the reported high concentration of $Mg^{2+}$ in amniotic fluid (0.4 mM) [32]. Inhibition could be overcome by the addition of 3 mM EDTA to the 105 000 $\times g$ supernatant fraction. The activity in the 105 000 $\times g$ pellet was unaffected by EDTA. When EDTA was added to amniotic fluid-700 $\times g$ supernatant, 1-alkylglycerol-3-$P$ hydrolysis was greatly increased (Figs. 4 and 5A). Bovine serum albumin also stimulated enzyme activity in this fraction (Fig. 5A), but was without effect on the 105 000 $\times g$ pellet. The highest activity in the 700 $\times g$ supernatant could be obtained with 3 mM EDTA in the absence of albumin, but in this case poor linearity with both time and protein concentration was observed. Therefore, for the 700 $\times g$ supernatant, the assay medium routinely contained 3.5 $\mu$mol of Tris/maleate buffer (pH 6.0), 50 nmol of 1-alkyl-[2-$^3$H]glycerol-3-$P$ (approx. 20 Ci/mol), 0.5 $\mu$mol of EDTA and 0.5 mg of bovine serum albumin, in a total volume of 100 $\mu$l. Under these conditions the assay was linear (Fig. 5B), and it was possible to determine phosphatidate phosphohydrolase activity in as little as 10 $\mu$l of amniotic fluid-700 $\times g$ supernatant.

TABLE I

THE DISTRIBUTION OF PROTEIN AND PHOSPHATIDATE PHOSPHOHYDROLASE IN HUMAN AMNIOTIC FLUID

Assays of phosphatidate phosphohydrolase were performed in 35 mM Tris/maleate buffer (pH 6.0) containing 0.5 mM 1-alkyl-[2-$^3$H]glycerol-3-$P$ (15 $\mu$Ci/mol) and 65 $\mu$g protein, in the absence of EDTA and bovine serum albumin. Incubations were for 20 min. The results shown are from a typical experiment. Absolute values vary with the gestational age of the amniotic fluid. n.d., not determined.

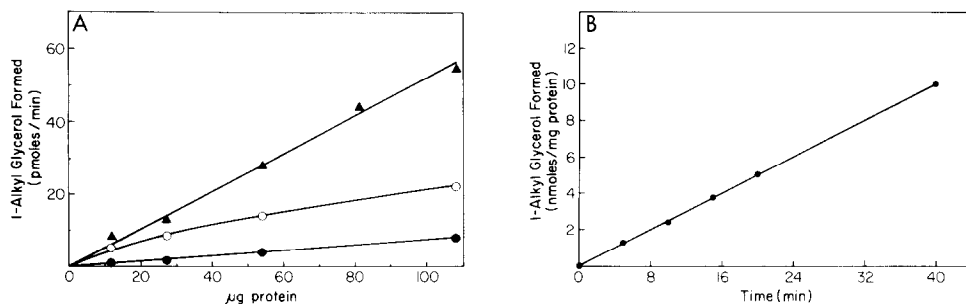| Fraction | Phosphohydrolase activity | | Total protein (mg) |
|---|---|---|---|
| | nmol 1-alkylglycerol/ min per mg protein | Total activity (%) | |
| Whole amniotic fluid | 0.29 | 100.0 | 8.16 |
| 700 $\times g$ supernatant | 0.26 | 86.5 | 7.87 |
| 700 $\times g$ pellet | n.d. | n.d. | n.d. |
| 105 000 $\times g$ supernatant | 0.06 | 19.5 | 7.68 |
| 105 000 $\times g$ pellet | 10.21 | 185.5 | 0.43 |

Fig. 5. (A) The dependence of 1-alkylglycerol-3-$P$ hydrolysis on protein concentration. Three assay systems are compared. In the first (●), the assay consisted of 3.5 $\mu$mol Tris/maleate buffer (pH 6.0), 50 nmol 1-alkyl-[2-$^3$H]glycerol-3-$P$ (20 Ci/mol) and amniotic fluid-700 × $g$ supernatant (80 $\mu$g protein). The second medium (○) contained in addition, bovine serum albumin (5 mg/ml), while the third (▲) included both albumin (5 mg/ml) and EDTA (5 mM). Incubations were for 45 min. Mean of three determinations. (B) The time course of 1-alkylglycerol-3-$P$ hydrolysis by amniotic fluid-700 × $g$ supernatant. Assays were at 37°C for the times indicated and contained, 3.5 $\mu$mol Tris/maleate buffer (pH 6.0), 50 nmol 1-alkyl-[2-$^3$H]glycerol-3-$P$ (20 Ci/mol), 0.5 $\mu$mol EDTA, 0.5 mg bovine serum albumin and amniotic fluid-700 × $g$ supernatant (65 $\mu$g protein) in a total volume of 100 $\mu$l. Mean of six determinations.

*Contribution of acid and alkaline phosphatases to 1-alkylglycerol-3-P hydrolysis*

In an attempt to determine the specificity of the assay procedure, the ability of non-specific phosphatases to hydrolyze 1-alkylglycerol-3-$P$ was investigated. At pH 5.6, the 700 × $g$ supernatant showed little activity with $p$-nitrophenyl-phosphate as substrate and conversely purified acid phosphatase from potato did not hydrolyze 1-alkylglycerol-3-$P$ (Table II). This result, combined with the observed optimum at pH 6.0, argues against 1-alkylglycerol-3-$P$ hydrolysis by amniotic fluid being attributable to acid phosphatase. At pH 10.0, both the 700 × $g$ supernatant and the 105 000 × $g$ pellet rapidly hydrolyzed $p$-nitro-

TABLE II

THE ESTIMATION OF ACID AND ALKALINE PHOSPHATASES IN HUMAN AMNIOTIC FLUID

The results shown are the means of three determinations performed on a 700 × $g$ supernatant prepared from three pooled amniotic fluids (mean phosphatidylcholine/sphingomyelin = 1.8). Assays were described in the text using the following buffers; 100 mM sodium citrate (pH 5.6), 35 mM Tris/maleate (pH 6.0), 40 mM 2-amino-2-methyl-propan-1-ol (pH 9.5) and 150 mM 2-amino-2-methyl-propan-1-ol (pH 10.0). n.d., not determined.

| Enzyme system | Enzyme activity (nmol/min with mg per protein) | |
|---|---|---|
| | 1-alkyl-glycerol-3-$P$ | $p$-Nitrophenyl-phosphate |
| Acid phosphatase (potato), pH 5.6 | 0 | 194 |
| Alkaline phosphatase (calf intestine), pH 10.0 | 20 250 | 600 000 |
| Alkaline phosphatase (calf intestine), pH 10.0 + 5 mM EDTA | 0 | 13 500 |
| 700 × $g$ supernatant, pH 5.6 | 0.07 | <0.05 |
| 700 × $g$ supernatant, pH 6.0 | 0.08 | n.d. |
| 700 × $g$ supernatant, pH 6.0, + 5 mM EDTA | 0.66 | n.d. |
| 700 × $g$ supernatant, pH 9.5 | 0.02 | 8.3 |
| 700 × $g$ supernatant, pH 9.5, + 5 mM EDTA | 0.18 | 1.6 |

phenylphosphate, indicating the presence of significant amounts of alkaline phosphatase (Table II). However, a number of observations suggest that it is not alkaline phosphatase which is responsible for 1-alkylglycerol-3-$P$ hydrolysis by amniotic fluid at pH 6.0. First of all, the rate of hydrolysis of 1-alkylglycerol-3-$P$ at pH 9.5 is only 45% of that at pH 6.0 (Fig. 1). Secondly, with purified alkaline phosphatase from calf intestine, 1-alkylglycerol-3-$P$, is a relatively poor substrate compared with $p$-nitrophenylphosphate (Table II). Finally, while calf intestine alkaline phosphatase with either $p$-nitrophenylphosphate or 1-alkylglycerol-3-$P$ as substrate is greatly inhibited by 5 mM EDTA, for amniotic fluid only $p$-nitrophenylphosphate hydrolysis is inhibited. 1-Alkylglycerol-3-$P$ hydrolysis is stimulated by the same concentration of EDTA at either pH 6.0 or pH 9.5 (Table II, Fig. 4).

*Relationship between 1-alkylglycerol-3-P hydrolysis by amniotic fluid and the phosphatidylcholine/sphingomyelin ratio*

Johnston and co-workers [14] have described the relationship between phosphatidate phosphohydrolase and the phosphatidylcholine/sphingomyelin ratio in amniotic fluid during gestation. Experiments here were undertaken to determine if the assay procedure described above, using 1-alkylglycerol-3-$P$, could form the basis of a rapid, sensitive clinical assay for phosphatidate phosphohydrolase in amniotic fluid. It was of particular interest to see how 1-alkylglycerol-3-$P$ hydrolase activity varied when the phosphatidylcholine/sphingo-
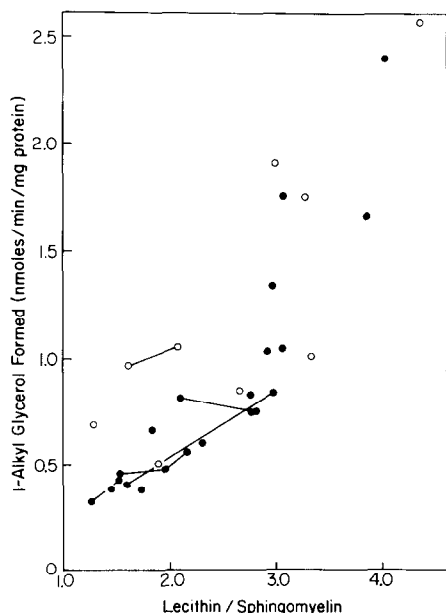


Fig. 6. The relationship between phosphatidate phosphohydrolase and the phosphatidylcholine/sphingomyelin ratio of amniotic fluid. Enzyme activity was measured in 700 X $g$ supernatants, using 1-alkylglycerol-3-$P$ as substrate in the presence of bovine serum albumin (5 mg/ml) and EDTA (5 mM) as described under Materials and Methods. Data points from samples of amniotic fluid taken at different times from the same patient are joined by lines. Values are means of duplicate determinations. (●, nondiabetics; ○, type B or C diabetics).

myelin ratio was in the region of 2.0. This ratio has been used widely as the lower limit when predicting fetal lung maturity. The data in Fig. 6 were obtained using $700 \times g$ supernatant fractions of a series of amniotic fluids. In general, each patient contributed one sample of amniotic fluid. Where serial samples were taken from the same patient, these data points are joined by lines. The results indicate a positive correlation between 1-alkylglycerol-3-$P$ hydrolysis and the phosphatidylcholine/sphingomyelin ratio.

## Discussion

It has been shown that the prenatal surge in surfactant synthesis is preceded by increased levels of phosphatidate phosphohydrolase in the lung [9]. Phosphatidate phosphohydrolase appears to be secreted into the lung alveoli along with the surfactant in the form of lamellar bodies and is found in increasing amounts in amniotic fluid as gestation proceeds [14]. In amniotic fluid, the enzyme is probably still associated with lamellar bodies [13,31]. Jimenez and Johnston [13] have suggested that the measurement of amniotic fluid phosphatidate phosphohydrolase may be a valid alternative to estimating the phosphatidylcholine/sphingomyelin ratio for assessment of fetal lung development. In initial experiments, we found that in contrast with the brain enzyme, membrane-bound phosphatidate was not a good substrate for amniotic fluid phosphatidate phosphohydrolase and that hydrolysis of aqueous dispersions of phosphatidate proceeded with a low affinity but with velocities similar to those previously reported [14]. A water-soluble substrate analog (1-alkylglycerol-3-$P$) has been used to develop a rapid, sensitive assay in guinea pig brain [18]. The present study was undertaken to determine whether 1-alkylglycerol-3-$P$ is suitable for a convenient clinical assay and for further characterization of amniotic fluid phosphatidate phosphohydrolase.

By several criteria, hydrolysis of both the synthetic substrate (Table I) and phosphatidate [13] is attributable to the same enzyme(s) in amniotic fluid. The amniotic fluid-$105\,000 \times g$ pellet is enriched in both phosphohydrolase activities, and heat denaturation curves are superimposable. Using either substrate, the enzyme preparation was relatively heat resistant and the non-linearity of denaturation (inset, Fig. 3) suggested the presence of several catalytic proteins, one of which was heat stable. A heat-stable alkaline phosphatase arising from trophoblast cells in the placenta has been detected in amniotic fluid [30], and the possibility that this accounted for the heat-stable component was investigated. The pH profile (Fig. 1), however, showed an optimum at pH 6.0. The secondary peak at pH 9.5 is not explained but would be unlikely to contribute significantly to the assay at pH 6.0. It is difficult to attribute even the activity at pH 9.5 to alkaline phosphatase since, like hydrolysis at pH 6.0, it is stimulated by EDTA while alkaline phosphatase is inhibited by EDTA. Parenthetically, a further advantage of the synthetic analog is its alkaline stability. This not only permitted phosphatidate phosphohydrolase activity measurement at high pH, but also facilitated quantitative extraction of the reaction product, neither of which was feasible using substrates with acyl groups.

When phosphatidate phosphohydrolase was assayed in amniotic fluid-700 ×

*g* supernatant using 1-alkylglycerol-3-*P* as substrate, enzyme activity was found to increase with the phosphatidylcholine/sphingomyelin ratio (Fig. 6) in agreement with earlier work in which phosphatidate was the substrate [35]. The exact relationship between these two clinically useful indicators of fetal lung maturation is unclear. If de novo synthesis of surfactant phosphatidylcholine in lung is limited by the availability of diacylglycerol, increased levels of phosphatidate phosphohydrolase could result in an overall stimulation of this biosynthetic pathway. It is thought that increased phosphatidate phosphohydrolase activity in amniotic fluid toward the end of gestation reflects increased levels of this enzyme in type II alveolar epithelial cells in the lungs of the fetus. Lung surfactant is secreted from these cells in the form of lamellar bodies and phosphatidate phosphohydrolase has been shown histochemically to be associated with these structures [37]. There is indirect evidence that the enzyme is secreted along with lamellar bodies and that fetal lung is the major source of amniotic fluid phosphatidate phosphohydrolase [13]. Even though most of the increased phosphatidate phosphohydrolase in amniotic fluid appears to be due to increased secretion of the enzyme from fetal lungs, other causes of the increased activity cannot be ruled out. For example, the composition of amniotic fluid is changing greatly over this period of gestation [38], and the removal of an inhibitor or appearance of an activator of phosphatidate phosphohydrolase could produce the same results. The concentration of phosphatidylcholine rises from 3 to 9 mg/100 ml during the period studied here [38], and its bearing on the measurement of phosphatidate phosphohydrolase was considered. We found, in fact, that added synthetic dipalmitoyl phosphatidylcholine was somewhat stimulatory, but could not account for the observed 8-fold increase in phosphohydrolase activity (Fig. 6) even when 50 mg/100 ml were added.

A number of pathological conditions are known to influence the phosphatidylcholine/sphingomyelin ratio and hamper evaluation of fetal lung development [38,39]. Of these conditions, maternal diabetes is of greatest clinical concern. Mild diabetes (Class B, C) delays both lung development and the increase in the phosphatidylcholine/sphingomyelin ratio [39]. The preliminary studies reported here with the new assay indicate that these patients may have higher phosphatidate phosphohydrolase levels than nondiabetics with equivalent phosphatidylcholine/sphingomyelin ratios. When enzyme activity is plotted against gestational age, these patients again appear to have higher levels of enzyme at a particular time in gestation than do nondiabetics. This would suggest that the explanation for the delayed phosphatidylcholine/sphingomyelin ratio in these patients does not lie in a deficiency of phosphatidate phosphohydrolase.

It is not known at present whether the enzyme demonstrates varying affinities for the stereoisomers of phosphatidate, 1-acylglycerol-3-*P*, or 1-alkylglycerol-3-*P*. We interpret the observed high *V* of the synthetic racemic analog to be largely due to its greater solubility than phosphatidate. This does not however explain its low $K_m$, and raises the question of whether phosphatidate is the true physiological substrate for the enzyme. Johnston et al. [40], have suggested that the substrate may be phosphatidylglycerol phosphate. The enzyme then would catalyze the final reaction in the biosynthesis

of phosphatidylglycerol, a lipid which has recently received considerable attention as a component of lung surfactant [41—43]. Regardless of the nature of the physiological substrate, the enzymic hydrolysis of 1-alkylglycerol-3-*P* appears to be a useful indicator of fetal lung maturity, and it is hoped that the new assay described here will complement existing diagnostic procedures [44].

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