diatr. Res. *14*: 891-893 (1980)

PHOSPHATIDIC ACID PHOSPHOHYDROLASE AND PHOSPHOLIPIDS IN TRACHEAL AND AMNIOTIC FLUIDS DURING NORMAL OVINE PREGNANCY

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SUMMARY

Samples of amniotic fluid and fetal tracheal fluid were obtained from 36 ovine nancies which were studied either acutely or chronically during the last two-thirds of ation (65 to 149 days). The specific activity of phosphatidic acid phosphohydrolase Pase), disaturated lecithin (DS-L), total phospholipids (TPL), and the L/S ratio were yed in the amniotic and tracheal fluids. There was a significant and progressive ease in the specific activity of PAPase in tracheal fluid beginning after 110 days, easing from 66 ± 7 nmoles phosphate released x mg^{-1} protein x $hour^{-1}$ (Mean ± S.E.M.) 0 days, to 107 ± 6 at 111 to 120 days, and to 277 ± 70 at 131 to 144 days. The rise in ase specific activity was followed by a parallel rise in the DS-L fraction of the TPL -L/TPL ratio), increasing from a DS-L/TPL ratio of 0.06 ± 0.01 in fetuses ≤ 120 days, .29 ± 0.06 at 121 to 130 days, and to 0.50 ± 0.18 at 131 ± 135 days. The PAPase ific activity and the L/S ratio in amniotic fluid did not change during pregnancy.

SPECULATION

Neonatal respiratory distress syndrome is the result of inadequate production of ace active material by the fetal and neonatal lung. The enzyme PAPase occupies a ral role in the biosynthesis of the glycerophospholipids, and increases in the specific vity of PAPase in human amniotic fluid and in fetal rabbit lung tissue precede or are llel with the increase in the L/S ratio and pulmonary surfactant synthesis, ectively. We have shown that this sequence is also demonstrable in ovine fetal heal fluid, thus providing an animal preparation in which the formation and lation of surfactant biosynthesis during fetal lung maturation can be investigated in .

INTRODUCTION

A deficiency in the quantity or composition of pulmonary surfactant is believed e the cause of neonatal respiratory distress syndrome (3). Although the biosynthetic ways for the production of the glycerophospholipids of surfactant have been well mented in recent years, the mechanism(s) by which surfactant production is ulated during fetal maturation is an enigma. Based on the results of studies ducted in our laboratories and others, it is likely that phosphatidic acid phospho-rolase, PAPase (EC3.1.3.4) occupies a central role in the regulation of the rate of erophospholipid biosynthesis (11,12,13,22). PAPase catalyzes the hydrolysis of phatidic acid giving rise to the formation of sn 1,2 diglycerides (19,23), the co-trate for phosphatidylcholine (lecithin) biosynthesis. It is also likely that PAPase lyzes the hydrolysis of phosphatidylglycerophosphate, giving rise to phosphatidyl-erol (14). Thus PAPase may be important in the biosynthesis of phosphatidylcholine phosphatidylglycerol, the two glycerophospholipids which account for nearly 90% of ophospholipids of lung surfactant. Moreover, these two glycerophospholipids are the t active of the surface-active components of surfactant.

Inasmuch as the pregnant ewe and her fetus can provide a chronically rumented animal model for the investigation of fetal and reproductive physiology and e is a paucity of information on the relationship between the specific activity of ase and the rate of release of glycerophospholipids into ovine fetal tracheal fluid and iotic fluid, the present studies were conducted. The purposes of the present stigation were 1) to develop a chronically instrumented fetal preparation for the y of ovine lung development during the last two-thirds of gestation, 2) to evaluate sequential changes in PAPase specific activity in ovine fetal tracheal fluid and in iotic fluid, and 3) to characterize the relationship between PAPase specific activity the glycerophospholipid composition in ovine fetal tracheal fluid and amniotic fluid.

MATERIALS AND METHODS

Thirty-six pregnant ewes of mixed Western breed and accurate gestational ages, ing from 65 to 149 days (term 145 to 150 days), were included in this study. Samples mniotic and tracheal fluids were obtained from 16 animals at the time of surgery or sacrifice at the completion of a study. Twenty animals were studied as chronic l preparations for an average of 11 days, range 2 to 31 days. Samples of amniotic were obtained from this group of animals only at the time of surgery, whereas heal fluid was sampled serially until the termination of pregnancy either by the ral onset of parturition or elective operative delivery. The animal preparation is ribed below.

ical Procedures

Fourteen of the 20 animals studied chronically underwent surgery at 106 to 112 , 2 at approximately 80 days, and 4 after 120 days of gestation. The animals were ed for 48 hours before surgery, but had access to water ad libitum. On the day of ery they were sedated with pentobarbital (5-10 mg/kg) administered via a utaneously placed jugular catheter, then blindfolded. Spinal anesthesia (8-10 mg acaine hydrochloride), supplemented with intravenous pentobarbital, was employed surgery. The animals were placed on the operating table in the supine position. A ine incision was made; and the uterine horn containing a singleton fetus and one ine horn in twin gestations was delivered onto the abdomen and wrapped in warm, t towels. The head of the fetus was located and the cricoid cartilage identified and ght into apposition with the uterine wall such that it was between the visible uterine d vessels. With blunt and sharp dissection the uterine wall and fetal membranes were ed. A 13 to 14 ml sample of amniotic fluid was obtained at this time and placed on The fetal trachea was palpated and a 2 cm incision was made over and parallel with With blunt dissection the fetal trachea was isolated, incised and a 13 to 14 ml sample racheal fluid obtained and placed on ice. A telfon catheter (1.2 mm i.d. by 2.2 mm was placed in the trachea to a point proximal to the bifurcation and secured with ilical tape; this also ligated the trachea, isolating the pulmonary compartment. The heal catheter was attached to a soft plastic bag of 300 ml capacity that would ide a reservoir for the collection of tracheal fluid. There was a second catheter ched to the bag that was exteriorized and used for sampling. The fetal skin was ed with 3-0 silk suture and the plastic bag placed in the amniotic sac. The uterine was closed with interlocking 3-0 silk suture. A second uterine incision was made in manner previously described (21) and siliconized polyvinyl catheters (0.77 mm i.d. by mm o.d.) were placed in a fetal femoral artery and vein and a branch of the umbilical . A sufficient length of catheter was placed in the amniotic sac to allow free fetal ement. Ampicillin, 250 mg, was injected into the amniotic cavity and the uterine was closed as described above. The catheters were sutured to the uterine wall for tion.

An electromagnetic flow probe (26) was placed around the uterine artery on the side of the uterus containing the instrumented fetus (20). Fetal catheters and flow probe leads were brought out of the ewe's abdomen through a single stab wound and were secured to the rectus fascia with 1-0 silk suture. The rectus sheath was closed. Additional polyvinyl catheters were inserted into a maternal femoral artery and vein. All catheters and flow probe leads were carried to the flank through a subcutaneous tunnel and stored in an external canvas pouch which was attached to the skin with steel pins. The skin incisions were closed with Michel clips. The catheters were flushed daily with heparinized saline containing 250 units/ml for maternal catheters and 500 units/ml for fetal catheters. Penicillin (600,000 units) and streptomycin (0.5 gm) were given to the mother on the day of surgery and for the next two days. The fetus received 50 mg of Ampicillin intravenously every other day throughout each experiment.

Experimental Protocol

In the chronic preparations the condition of the fetus was evaluated by monitoring heart rate, hematocrit, arterial blood gases and pH, and blood pressure within 3 to 4 hours after surgery, the first day postoperative, and every other day thereafter. Maternal heart rate, systemic blood pressure and arterial blood gases and pH were monitored in a similar manner. Uterine blood flow was monitored daily for 15 minutes.

The plastic tracheal fluid collection bag was emptied every 24 hours by gentle suction with a sterile 50 ml syringe. The sample was collected in ice and divided into 30 ml aliquots that were centrifuged immediately at 700 x g for 5 minutes. The supernatant fractions were removed and frozen immediately at -18°C in 10 and 30 ml aliquots until the time of assay. All other samples of tracheal and amniotic fluid were processed in a similar manner.

Assays

The phospholipids were extracted from 5 ml aliquots of amniotic and tracheal fluids according to the procedure of Gluck, et al. (8). The acetone-precipitated fraction was dissolved in chloroform, applied to silica HR thin layer chromatograms, and the phospholipids separated employing the solvent system chloroform:methanol:acetic acid:water (125:75:20:10). Following chromatography, the thin-layer plates were dried in air and the phospholipids were localized by exposure to iodine vapors. The phospholipid was quantified by the method of Parker and Peterson (17) as modified by Jimenez, et al. (12).

The specific activity of PAPase was determined in samples of amniotic and tracheal fluid by assaying the release of ^{32}P orthophosphate from ^{32}P phosphatidic acid employing the procedure described by Spitzer and Johnston (24). This method has been shown to be reliable for the measurement of PAPase activity in human amniotic fluid and gastric aspirates (10).

RESULTS

The mean specific activity of PAPase at various stages of gestation in 58 samples of fetal tracheal fluid and 55 samples of amniotic fluid are presented in Figure 1. The specific activity of PAPase in amniotic fluid did not change significantly throughout pregnancy, the mean value being 57.2 ± 6.10 (± S.E.M.) nmoles PO_4 released·mg^{-1} protein·hr^{-1}. On the other hand the specific activity of PAPase in fetal tracheal fluid began to rise after 111 days, increasing from a mean of 66 nmole PO_4 released·mg^{-1} protein·hr^{-1} between 101 to 110 days to 107 at 111 to 120 days ($p < 0.05$), reaching a value of 277 at 131 to 140 days gestation ($p < 0.001$). In 34 instances paired and simultaneously obtained samples of amniotic fluid and fetal tracheal fluid were available. Twenty nine of the paired samples (85%) were obtained after 111 days gestation. The values for PAPase specific activity obtained from these samples are presented in Figure 2. There is a nearly 3-fold higher specific activity in tracheal fluid than in amniotic fluid, 169 ± 24 (Mean ± S.E.M.) and 63 ± 8.2, respectively ($p < 0.001$, paired t test).

In order to describe the changes in the composition of the glycerophospholipids in ovine fetal tracheal fluid we measured the concentrations of disaturated lecithin (DS-L) and total phospholipid (TPL) in 55 samples. These data are presented in Figure 3 as the ratio of the concentrations of DS-L and TPL and are plotted as a function of gestational age in days. There is a sharp rise in the DS-L/TPL ratio that occurs between 121 and 130 days, increasing from a mean value of 0.07 at < 110 days to 0.29. By 131 to 135 days the mean ratio is 0.50. We were able to compare this to the L/S ratio determined on 36 amniotic fluid samples obtained from 65 to 149 days of gestation. In contrast to the rise in the DS-L/TPL ratio found in fetal tracheal fluid, there was no change in the amniotic fluid L/S ratio; the mean value (± S.E.M.) during pregnancy was 0.60 ± 0.05.

In Figure 4 the cumulative data for fetal tracheal PAPase specific activity and DS-L/TPL ratio are presented to illustrate their relationship during normal ovine pregnancy. The rise in PAPase specific activity preceeds or is concomitant with a parallel rise in the DS-L/TPL ratio, the former rising after 111 days gestation and the latter after 120 days gestation. This relationship is illustrated further in a chronically instrumented fetus studied longitudinally from 119 to 141 days gestation and is presented in the insert in Figure 4, each point representing the average value for a 5 day period. As observed in the cumulative data there are parallel increases in PAPase specific activity and the DS-L/TPL ratio in the fetal tracheal fluid, the rise in the former preceding that of the latter.

DISCUSSION

The chronically instrumented pregnant sheep employed in this investigation provide an excellent animal model for the study of reproductive physiology in a near physiologic state (20,21). The lamb fetus can be instrumented and studied in a "nonstressed" state (21). Such an animal model provides a means whereby one can sample various maternal or fetal compartments, e.g., blood, amniotic fluid, etc., for extended periods. It was the purpose of this investigation to develop a chronically instrumented fetal-maternal preparation using the pregnant sheep and to determine the usefulness of this model in the study of lung maturation. The stability of this chronic preparation is illustrated by the data presented in the insert in Figure 4. In this study we were able to maintain the instrumented sheep preparation for 23 days, terminating the pregnancy by elective operative delivery of a live fetus.

Previously we observed that the specific activity of PAPase in fetal rabbit lung tissue and in human amniotic fluid increased at or before the time of augmented surfactant synthesis (10,12,22). These findings were suggestive that the metabolism of phosphatidic acid occupies a central position in de novo pathways of phopholipid biosynthesis in lung tissue as well as other tissues (25). It also was found that the specific activity of PAPase in the gastric aspirate or nasopharyngeal secretions of neonates at birth was greater than that in samples of amniotic fluid obtained from the same infants, a finding that provided strong evidence that the fetal tracheal fluid, i.e. fetal lung, is the source of the PAPase activity found in human amniotic fluid (10,12). In the present study we found that the specific activity of PAPase in ovine fetal tracheal fluid increased dramatically after 110 days of gestation; that is, after the completion of 80% of pregnancy. This time course is similar to that observed in human amniotic fluid, where PAPase specific activity rises at 32 to 34 weeks, also after the completion of 80% of pregnancy (10,12). In contrast to the results of human studies, however, there was no significant alteration in the specific activity of PAPase in ovine amniotic fluid over the course of pregnancy studied. None the less, the finding that the specific activity of PAPase in ovine tracheal fluid in late pregnancy was three times that of PAPase in

amniotic fluid is similar to the relationship found between the PAPase activity in nasopharyngeal fluid and amniotic fluid in the human (12). This provides further support for the view that the source of amniotic fluid PAPase activity in the human is the fetal lung.

In human amniotic fluid (10,12) and fetal rabbit lung (24) the changes in the specific activity of PAPase are related in time to the synthesis of surface-active lecithin inasmuch as the rise in specific activity of PAPase enzyme precedes the lecithin surge or the rise in the L/S ratio. In the sheep the amniotic fluid L/S ratio, like the amniotic fluid PAPase activity, did not rise as pregnancy advanced, an observation also made by Rethmeier and Egbert (19). These findings, like those of the amniotic fluid PAPase activity, are supportive of the view that ovine amniotic fluid is not reflective of fetal tracheal secretions, in contrast to the human, and, as noted by Adams, et al. (2), is suggestive that in this particular species the majority of fetal tracheal fluid produced is swallowed. The findings in the ovine fetal tracheal fluid, however, are very similar to those noted in fetal rabbit lung and human amniotic fluid. After 120 days gestation there is a remarkable surge in the concentration of DS-L, expressed as the DS-L/TPL ratio, that followed closely and was parallel with the rise in PAPase activity (Figure 4). This surge in the DS-L/TPL ratio in fetal tracheal fluid occurred at the same time in pregnancy as the previously reported increases in surfactant activity in ovine fetal tracheal fluid and lung tissue homogenate when expressed as either disaturated lecithin concentration (6,23), surfactant flux (16), or surface tension (1,14). The intimate relationship betwen PAPase and the DS-L/TPL ratio in ovine fetal tracheal fluid is documented further by the results of the longitudinal study (insert Figure 4). As noted earlier regarding PAPase activity, the change in the DS-L/TPL ratio occurs after the completion of 80% of normal ovine pregnancy, the same time in human pregnancy when the amniotic fluid PAPase activity and L/S ratio increase. Thus, the findings of this study are supportive of the conclusion that the PAPase activity in tracheal fluid is a reflection of the increase in phospholipid synthesis occurring in fetal lung tissue; that the developmental characteristics of ovine fetal lung may be similar to those of the human; and finally, that the chronically instrumented animal model employed in these experiments may be useful in further evaluation of factors influencing fetal lung development.

Further studies now are necessary to evaluate the changes in the composition of surfactant that normally occur among the individual phospholipids in ovine fetal tracheal fluid, especially in view of recent observations that are supportive of an important role for phosphatidylglycerol in lung maturation (7,9).

REFERENCES AND NOTES

1. Adams, FH, and Fujiwara, T: Surfactant in fetal tracheal fluid. J. Pediat. 63:537 (1963).

2. Adams, FH, Desilets, DT, and Towers, B: Control of flow of fetal lung fluid at the laryngeal outlet. Respir. Physiol. 2:302 (1967).

3. Avery, ME and Mead, J: Surface properties in relation to atelectasis and hyaline membrane disease. Am. J. Diseases Children 97:517 (1959).

4. Bartlett, GR: Phosphorus assay in column chromatography. J. Biol Chem. 234:466 (1959).

5. Delahunty, TJ, Spitzer, HL, Jimenez, JM, and Johnston, JM: Phosphatidate phosphohydrolase activity in porcine pulmonary surfactant. Am. Rev. Respir. Dis. 119:75 (1979).

6. Fujiwara, T, Adams, FH, Sipos, S, and El-Salawy, A: "Alveolar" and whole lung phospholipids of the developing fetal lamb lung. Am. J. Physiol. 215:375 (1968).

7. Godinez, RI, Sanders, RL, and Longmore, WJ: Phosphatidylglycerol in rat lung. I. Identification as a metabolically active phospholipid in isolated perfused rat lung. Biochemistry 14:830 (1975).

8. Gluck, L, Chez,RA, Kulovich, MV, et al: Comparison of phospholipid indicators of fetal lung maturity in the amniotic fluid of the monkey and baboon. Am. J. Obstet. Gynecol. 120:524 (1974).

9. Hallman, M, and Gluck, L: Development of the fetal lung. J. Perinat. Med. 5:3 (1977).

10. Herbert, WNP, Johnston, JM, MacDonald, PC, and Jimenez, JM: Fetal lung maturation: Human amniotic fluid phosphatidate phosphohydrolase activity through normal gestation and its relation to the lecithin/sphingomyelin ratio. Am. J. Obstet. Gynecol. 132:373 (1978).

11. Jimenez, JM, Schultz, FM, MacDonald, PC, and Johnston, JM: Fetal lung maturation. II. Phosphatidic acid phosphohydrolase in human amniotic fluid. Gynecol. Invest. 5:245 (1974).

12. Jimenez, JM, Schultz, FM, and Johnston, JM: Fetal lung maturation. III. Amniotic fluid phosphatidic acid phosphohydrolase (PAPase) and its relation to the lecithin/sphingomyelin ratio. Obstet. Gynecol. 46:588 (1975).

13. Johnston, JM, Porter, JC, and MacDonald, PC: The biosynthesis and regulation of surfactant formation, in Gatt, Freysz, and Maudel, editors: Enzymes of Lipid Metabolism, New York, 1978, Plenum Press, p. 327.

14. Kikkawa, Y, Motoyama, EK, and Cook, CD: The ultrastructure of the lungs of lambs. Amer. J. Pathol. 47:877 (1965).

15. Lowry, OH, Rosebrough, NJ, Farr, AL, and Randall, RJ: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265 (1951).

16. Mescher, EJ, Platzker, ACG, Ballard, PL, Kitterman, JA, Clements, JA, and Tooley, WH: Ontogeny of tracheal fluid, pulmonary surfactant, and plasma corticoids in the fetal lamb. J. Appl. Physiol. 39:1017 (1975).

17. Parker, F, and Peterson, NF: Quantitative analysis of phospholipids and phospholipid fatty acids from silica-gel thin layer chromotograms. J. Lipid Res. 6:455 (1965).

18. Ravinuthala, HR, Miller, JC, and Weinhold, PA: Phosphatidate phosphatase activity and properties in fetal and adult rat lung. Biochim. Biophys. Acta 530:347 (1978).

19. Rethmeier, HB, and Egberts, H: Transversal and longitudinal lecithin-sphingomyelin ratios in amniotic and lung fluids of fetal lambs. Am. J. Obstet. Gynecol. 122:593 (1975).

20. Rosenfeld, CR, Morriss, FH, Jr., Makowski, EL, Meschia, G, and Battaglia, FC: Circulatory changes in the reproductive tissues of ewes during pregnancy. Gynecol. Invest. 5:252 (1974).

21. Rosenfeld, CR, Worley, RJ, Milewich, L, Gant, NF, Jr., and Parker, CR, Ovine fetal-placental sulfoconjugation and aromatization of dehydroepiandrosterc Endocrinology. In Press.

22. Schultz, FM, Jimenez, JM, MacDonald, PC, and Johnston, JM: Fetal l maturation. I. Phosphatidic acid phosphohydrolase in rabbit lung. Gynecol. Inv 5:222 (1974).

23. Smith, BT, Worthington, D, and Piercy, WN: The relationship of cortisol cortisone to saturated lecithin concentration in ovine amniotic fluid and fetal lung liq Endocrinology 101:104 (1977).

24. Spitzer, HL, and Johnston, JM: Characterization of phosphatic phosphohydrolase activity associated with isolated lamellar bodies. Biochim. Biop Acta 531:275 (1978).

25. Vaurecka, M, Mitchel, MP, and Hubscher, G: The effect of starvation on incorporation of palmitate into glycerides and phospholipids of rat liver homogena Biochem. J. 115:139 (1969).

26. Micron Instruments, Inc., Los Angeles, California

27. This research was supported in part by NIH General Research Grant No. HD08783-06 and a Grant-in-aid from the Dallas Crystal Charity Ball.

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29. Received on October 23, 1979.

30. Accepted for publication February 5, 1980.

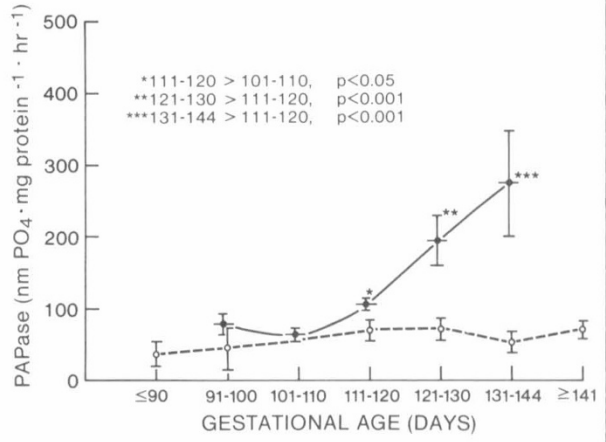


Figure 1. Changes in the specific activity of PAPase in amniotic (o) and fe tracheal (●) fluids during ovine pregnancy. The mean ± S.E.M. are presented. Analy was by unpaired t-test. For tracheal fluid samples n=4 at 91-100 days, n=5 at 101- days, n=34 at 111-120 days, n=9 at 121-130 days, and n=6 at 131-144 days.

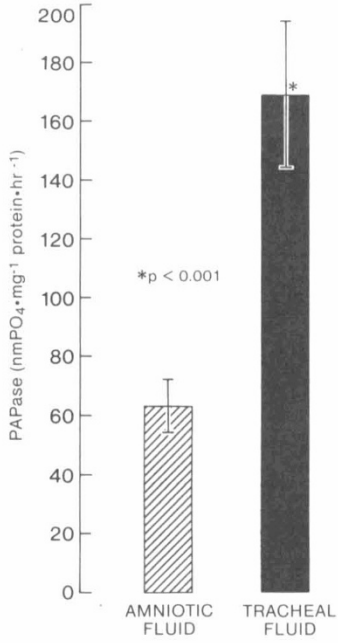
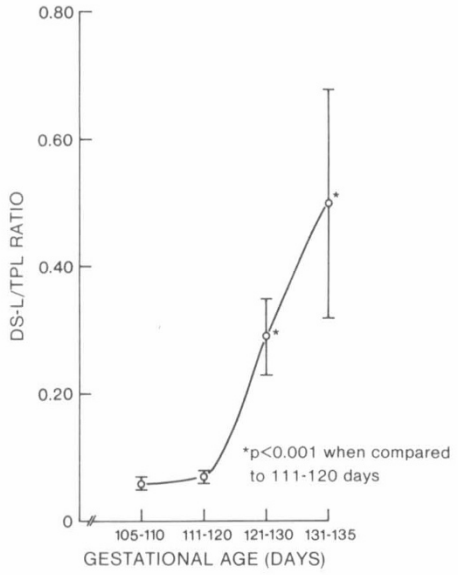


Figure 2. The difference in the specific activity of PAPase in 34 paired sample amniotic and fetal tracheal fluids (Mean ± S.E.M.).



ure 3. Changes in the ratio of disaturated lecithin to total phospholipid (DS-PL) in fetal tracheal fluid during ovine pregnancy (Mean ± S.E.M.). Analysis was by aired t-test. At 105-110 days n=6, 111-120 days n=29, 121-130 days n=16, and 131-135 s n=4.

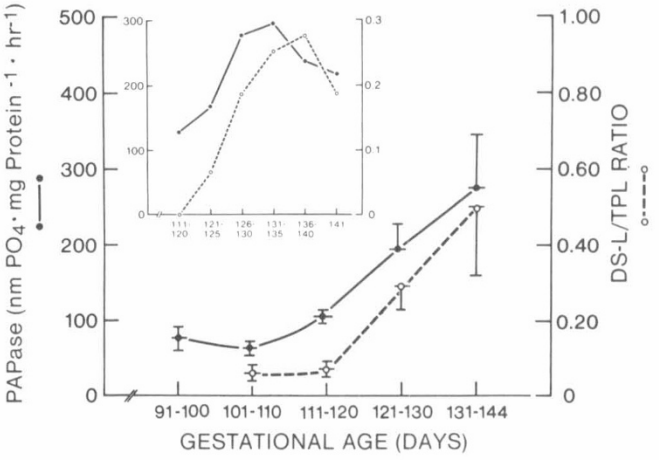


Figure 4. Relationship between the changes in PAPase specific activity and the ratio of disaturated lecithin to total phospholipid (DS-L/TPL) in fetal tracheal fluid during normal ovine pregnancy (Mean ± S.E.M.). Longitudinal data obtained from a chronically instrumented fetus, 119 to 141 days, are illustrated in the insert.

1-3998/80/1407-891$02.00/0

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