Chemical Changes in Amniotic Fluid*

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The scope of my talk as represented by its title encompasses a very large field which time does not allow me to cover in its entirety. Therefore, I will limit my remarks to one very specific area where chemical changes in amniotic fluid could be used to predict fetal maturity.

An ever present dilemma in modern obstetrical management is the proper timing of delivery of the fetus in situations where complications either threaten its own life *in utero* or significantly affect maternal morbidity. In such situations the obstetrician is confronted with the equivocal choice between premature delivery and the high risk to the fetus of continued intrauterine existence. In addition, he is placed in a unique position where any sort of direct communication with one of his "patients," namely the fetus, is nearly impossible. In the past, the delivery of obstetrical care to the fetus and the monitoring of its well-being were possible only through the agency of the maternal organism. To improve upon this situation, methods had to be found that would give a more direct and accurate reflection of fetal status. Hence, as complete a collection as possible of accurate indices relating to fetal maturity is of great importance to the obstetrician. Some of the notable complications of pregnancy in which these indices would be of value are diabetes, toxemia, erythroblastosis fetalis, and previous poor obstetrical history: for example, cases requiring repeat cesarean sections or abruptio placentae.

In the late fifties (Jeffcoate and Scott, 1959), it was recognized that the fetus contributed metabolic products and secretions to the amniotic fluid, and this encouraged many researchers to investigate a possible correlation between the status of the fetus and the amniotic fluid constituents. At that time, however, the technique for amniocentesis had not been

generally accepted, although it has been known for over two decades, and the progress of research was slow. With the acceptance of amniocentesis as an aggressive means of diagnosis, the procedure for withdrawal of amniotic fluid led to several investigations into fetal maturity indices. Amongst these were the measurements of amniotic fluid creatinine (Pitkin and Zwirek, 1967; Liley, 1961; Mandelbaum, *et al*, 1967), bilirubin (Droegemueller, 1969), and the examination of the cytology of cells stained with Nile Blue Sulfate (Brosens and Gordon, 1966; Anderson and Griffiths, 1968). These indices are now used in a fairly routine manner in the management of obstetrical patients, and I shall not go into any detail about them. A further and more specialized offshoot of this research has been the measurement of certain other specific amniotic fluid constituents that might lead, in the near future, to a more reliable picture of fetal well-being or distress in addition to fetal maturity.

In spite of all the advances that have been made, there still exists an appreciable degree of perinatal mortality today, the major contributor being respiratory distress due to premature delivery. An estimated 25,000 newborn deaths annually in the United States are attributed to this cause (Gluck, 1971). The stage of fetal lung maturity at the time of delivery is of prime importance as it most often entirely determines the survival or death of the neonate. For the neonate to survive, its lung has to assume immediate function with the change from an intra to an extrauterine environment. Thus, in the case of a pregnancy complicated, for example, by the presence of maternal diabetes or toxemia, the obstetrician has to walk a tightrope where he must maintain, to the best of his ability, a critical balance between neonatal death due to respiratory distress (hyaline membrane disease) and the threat of intrauterine demise caused by maternal disease. The frustrating decision that has to be made is, "When

is the earliest the obstetrician can deliver a healthy baby that will survive?"

At the moment, what seems to be a ray of hope on the horizon for the obstetrician has resulted from research that was started in the early fifties on the phospholipid content and its patterns in the fetal lung. As early as 1929, von Neergaard (von Neergaard, 1929), demonstrated that surface forces contribute significantly to the retractive pressure of the lung. At that time he postulated a lung coating to explain these observations. In 1955, Pattle (Pattle, 1955), showed that pulmonary edema foam contained some substance, or substances, that stabilized the tiny bubbles seen characteristically in such foam, and in 1957, Clements (Clements, 1957) and Avery and Mead (Avery and Mead, 1959) identified a surface tension lowering substance in lung tissue. These observations, subsequently confirmed by several workers, led to the conclusive identification of this surface tension lowering compound, called a surfactant, as being α,β-dipalmitoyl lecithin.

I am now going to highlight some of the significant laboratory findings that have led us to the point at which we are today. In 1961, Klaus and his co-workers (Klaus, et al, 1961) showed that bovine lung foam contained a mixture of several surface active compounds, the major constituent being dipalmitoyl lecithin. The other surface active compounds he found were sphingomyelin, phosphatidyl inositol, phosphatidyl dimethylethanolamine, and lysolecithin. The chemical structures of some of these compounds are depicted in Fig. 1. During the sixties, significant details of the surfactant system were gleaned through the work of Morgan (Morgan, et al, 1965), Fujiwara (Fujiwara, et al, 1968), and Gluck (Gluck, Kulovich, et al, 1967, 1970) all working independently. Morgan and his group worked on the identification of the various components of the surface active system in alveolar and whole lung homogenates in the dog. They found that of the total lipids found in dog lung, phospholipids constituted the major group at 74.1%. Of this group, phosphatidyl choline was the major component. Phosphatidyl ethanolamine, lysophosphatidyl choline, which is lysolecithin, and sphingomyelin were also found, but in much smaller quantities. These are all surface active along with dipalmitoyl lecithin or phosphatidyl choline, but phosphatidyl choline seems to be by far the major component. As seen in Fig. 1, the lecithins are characterized by fatty acid groups R_1 and R_2 on the α and β positions of the triglyceride backbone. Fujiwara and his associates
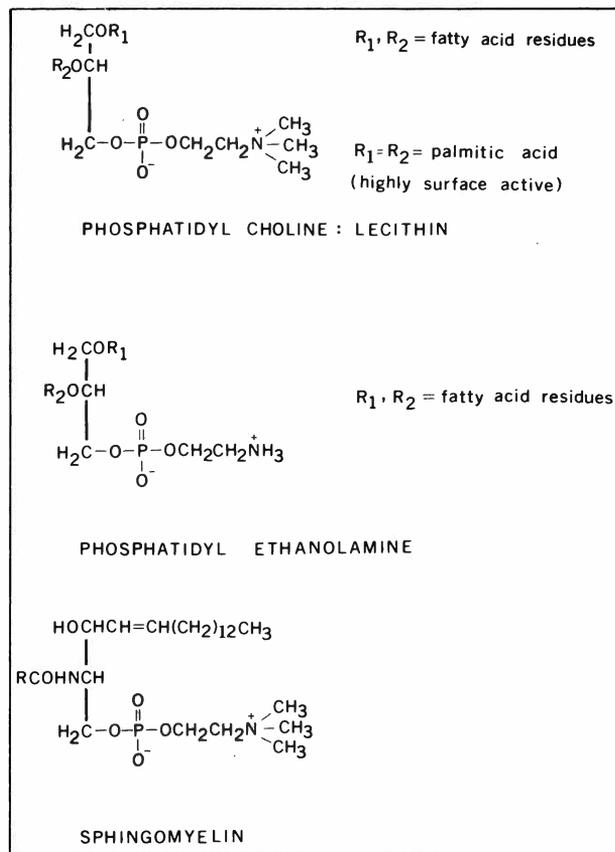

Fig. 1—Chemical structures of some phospholipids.

(Fujiwara, et al, 1968) reported on the significance of these fatty acids, which are normally C_{14} to C_{18} fatty acids, and the total concentration of individual phospholipids in the developing fetal lamb lung. He took fetuses at three different ages of gestation; immature (99–119 days), transitional (120–134 days) and term (135–145 days). Gestational age in sheep is about 140 days. He found that as gestation proceeded toward term, the amount of phospholipids, both on a weight basis and on a total phospholipid basis, increased. He also found that phosphatidyl choline increased while phosphatidyl ethanolamine decreased, and this will be an important factor when we talk about the biosynthetic pathways. The significance of the fatty acids was demonstrated by the observation that the percentage of saturated fatty acids in the whole molecule increased towards term, whereas the percentage of saturated fatty acids at the β position stayed fairly constant. This means that now we have two parameters by which the state of development of the fetal lung can be assessed; in general, the total amount of lecithin present and specifically, the

amount of lecithin esterified with saturated fatty acids. To test the hypothesis that lung maturity is reflected by an increase in saturated lecithins, and vice versa, Brumley and his colleagues measured the surface tension and phospholipid content of normal lungs and lungs from infants with respiratory distress syndrome (Brumley, et al, 1967). Brumley's data is shown in Table 1. As I men-

they parallel one another and continue to do so until about the 25th week of gestation where lecithin starts rising slightly more rapidly than sphingomyelin. At about the 30th to 34th week there is a sharp increase in lecithin concentration till term, whereas sphingomyelin plateaus and starts going down again. The reason for this dramatic rise in lecithin biosynthesis at about the 32nd to 34th week

TABLE 1

Phospholipid Analysis of Lung Tissue*

Condition	Phospholipid (mg/g wet weight)	Phosphatidyl choline (as % of total phospholipid)	Saturated fatty acids (as % in phosphatidyl choline)	Surface Tension (dynes/cm)
Infants with respiratory distress	10.4	46.4	64.7	23
Infants recovered from respiratory distress	17.4	56.9	70.4	3.0
Infants without respiratory distress	22.3	58.8	84.2	6.0
Normal adults	16.8	45.8	80.8	6.0

* Data adapted from Brumley, Hodson, and Avery, 1967.

tioned earlier, what the surfactant does is lower surface tension and thus produce more elasticity in the lung. In adults the surface tension of trachial aspirates is somewhat of the order of 6 dynes/cm., and the total phospholipid content is 16.8 mg/gm wet weight. The content of phosphatidyl choline, as a percentage of total phospholipids, is about 45.8%. In the infant with no respiratory distress syndrome these values are very close to those in the normal adult. With respiratory distress syndrome the surface tension of trachial aspirates becomes much higher. The amount of phospholipid is considerably lower compared to that of adults and infants with no respiratory distress syndrome, as are the amounts of phosphatidyl choline and saturated fatty acid. As the neonate recovers from the respiratory distress situation, these values slowly start returning to normal. This means that there is a valid justification for implicating the amount of phospholipid, specifically phosphatidyl choline, in the development of fetal lung. Gluck and his group (Gluck, 1971; Gluck, Kulovich, et al, 1967, 1970) reported extensively in the middle and late sixties on the biochemistry of surfactant production in the lung. They measured the amount of dipalmatoyl lecithin and sphingomyelin in amniotic fluid in humans (Gluck, 1971), with the results shown in Fig. 2. Gluck found that early in gestation, the amount of lecithin is slightly lower than that of sphingomyelin but that

can be partially explained by the picture of the biosynthetic pathways of these phospholipids (Fig. 3). The lecithins are synthesized mainly by two routes. In pathway 1 (the choline incorporation pathway) an α,β-di-substituted diglyceride reacts with cytidinediphospho choline (CDP-choline) thereby incorporating choline into the molecule which results in phosphatidyl choline, or lecithin. In pathway 2 (the methylation pathway) the α,β-diglyceride incorporates ethanolamine through the agency of cytidine-diphosphoethanolamine (CDP-ethanolamine) resulting in phosphatidyl ethanolamine (PE) which by subsequent methylations by S-adenosylmethionine gives phosphatidyl methyl ethanolamine (PME), phosphatidyl dimethyl ethanolamine (PDME), and finally lecithin. These two pathways need to be studied in greater detail than they have been, but certain facets about them are known. It has been shown that the dramatic surge in lecithin at the 33rd to 34th week appears because of the activation of pathway 1, that is, the choline incorporation pathway. Gluck has evidence, primarily clinical in nature, that the methylation pathway is the primary mode of lecithin biosynthesis in early gestation, but that on or after about the 34th week, the choline incorporation pathway becomes the more important (Gluck, 1971). He found that when he analyzed respiratory distress syndrome cases in prematures, most of them were acidotic and/or hypothermic. He also found that neither

acidosis nor hypothermia were associated with clinical respiratory distress syndrome in the full-term infant. When trachial aspirates were obtained from prenatal, or from premature infants while they were still hypothermic, he found little or no phosphatidyl dimethyl ethanolamine which meant that the methylation pathway was not functioning. As the infants were put into an incubator and temperature brought up, increasing amounts of PDME were found in trachial aspirates which indicated that the heat supplied to them was activating this pathway. These findings suggested that the methylation pathway is vulnerable to external insult. Yet if this were the case, then some full-term infants should also show respiratory distress, which they do not. Gluck's explanation for this is based on his evidence that early in gestation the methylation pathway is the more important but at about the 35th week the choline incorporation pathway takes over causing the surge of lecithin. As the choline incorporation pathway is not vulnerable to external insult, it is producing sufficient quantities of surfactant for the full-term infant not to have respiratory distress. Thus the premature infant is at a disadvantage because at around 30–34 weeks gestation when the methylation pathway is dominant and vulnerable to external insult, the choline incorporation pathway has not yet assumed maximal function, and hence, sufficient surfactant is not being produced. The only lab finding that Gluck reports in support of his hypothesis is that *in vitro*, the methylation pathway is markedly inhibited below a pH of 7.2; *in vivo*, exposure to cold or hypothermia or hypoxia causes acidosis in the neonate, which could then inhibit the methylation pathway.

With all this data, we now have the basis for developing a method for the prediction of re-
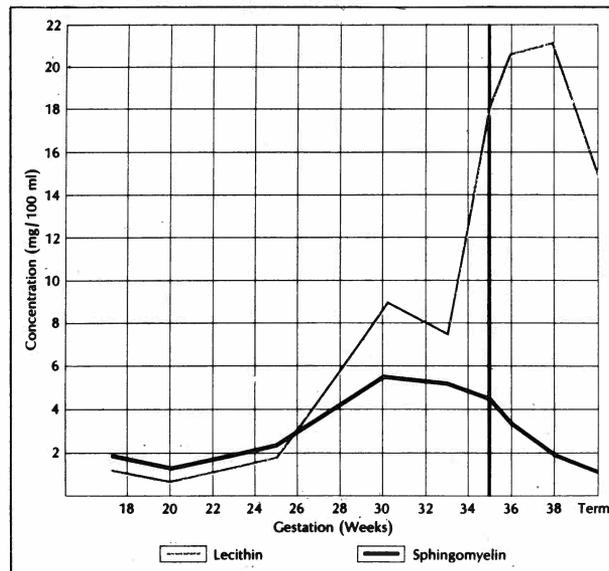

Fig. 2—Concentrations of sphingomyelin and lecithin in amniotic fluid during gestation. (Reprinted with permission from Gluck, *Hosp. Pract.* 6:45, 1971.)

spiratory distress in the neonate by measuring lecithin and sphingomyelin in amniotic fluid. Although lecithin and sphingomyelin are measured individually, the results are reported in terms of the ratio of lecithin to sphingomyelin. Once the ratio starts getting higher than 1 or 2, one might be able to give some indication about whether respiratory distress could be predicted in that fetus were it delivered at that time. The method we have developed and are using at present is a modification of Gluck's original method (Gluck, Kulovich, *et al,* 1971) and is outlined in Fig. 4. Three milliliters of amniotic fluid are taken to which are added 3 ml of methanol and 6 ml of chloroform in a centrifuge tube. The whole is extracted once, centrifuged, and the lower
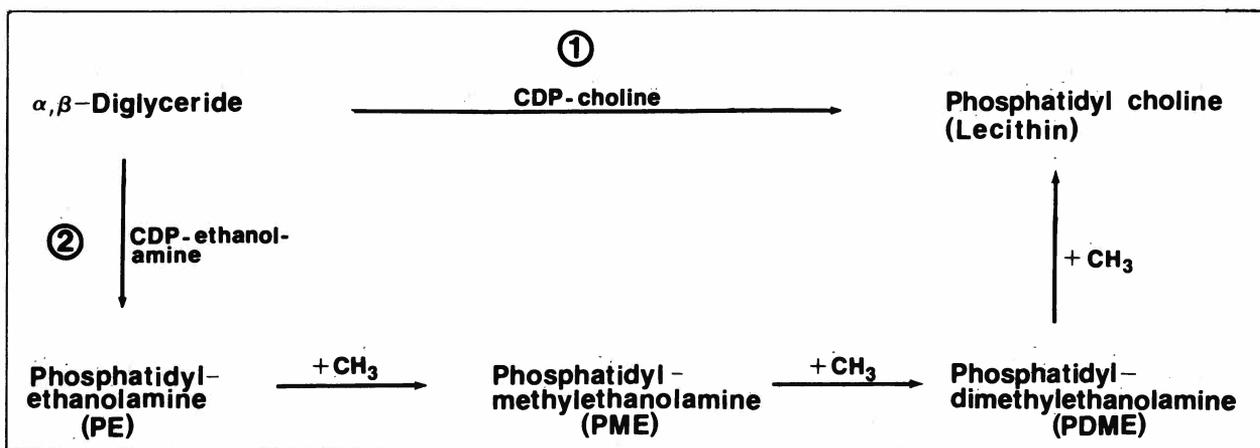

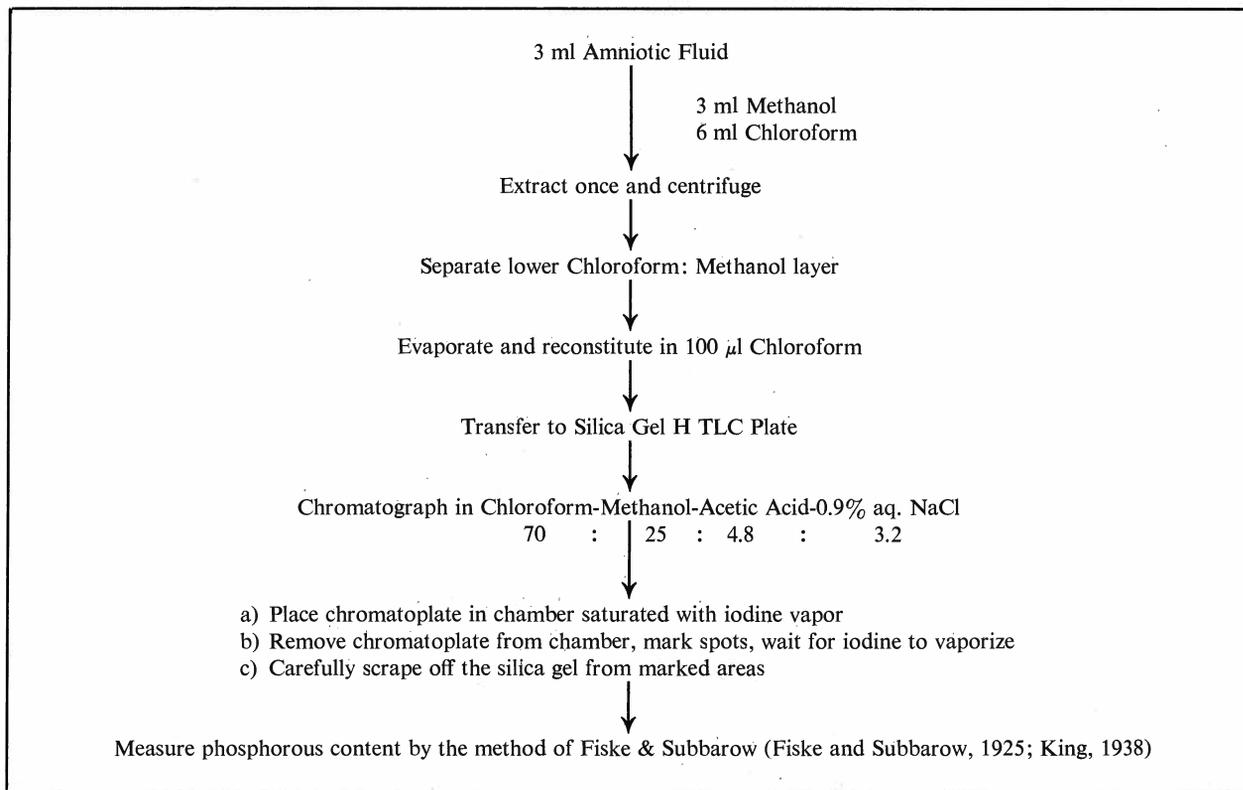Fig. 3—Two postulated pathways for lecithin biosynthesis.

3 ml Amniotic Fluid

3 ml Methanol
6 ml Chloroform

↓

Extract once and centrifuge

↓

Separate lower Chloroform: Methanol layer

↓

Evaporate and reconstitute in 100 μl Chloroform

↓

Transfer to Silica Gel H TLC Plate

↓

Chromatograph in Chloroform-Methanol-Acetic Acid-0.9% aq. NaCl
70 : 25 : 4.8 : 3.2

↓

a) Place chromatoplate in chamber saturated with iodine vapor
b) Remove chromatoplate from chamber, mark spots, wait for iodine to vaporize
c) Carefully scrape off the silica gel from marked areas

↓

Measure phosphorous content by the method of Fiske & Subbarow (Fiske and Subbarow, 1925; King, 1938)

Fig. 4—Methodologic flow sheet.

chloroform methanol layer is removed, evaporated, and reconstituted in 100 μl of chloroform. This is then transferred to a thin-layer plate which is run in the system, chloroform-methanol-acetic acid-0.9% aq. NaCl (70:25:4.8:3.2). We found this system to be better than the old chloroform-methanol-water ones which are normally used for phospholipids. Lecithin and sphingomyelin are visualized by placing the thin-layer plate in a chamber saturated with iodine vapor. The iodine is deposited reversibly on the areas where the lecithin and sphingomyelin are located. The spots are then marked, scraped off, and after the iodine has vaporized, the phosphorus content of the spots is measured by a modification of the Fiske and Subbarow method (Fiske and Subbarow, 1925; King, 1938). As one mole of phosphorus corresponds to one mole of lecithin and sphingomyelin, by measuring the amount of phosphorus, we can calculate the absolute amounts of lecithin and sphingomyelin and hence get the ratio of one to the other. The test we have developed takes an average technician approximately 3 hours for 6 quantitative determinations. For a quick but qualitative result, the test can be shortened by eliminating the quantitative step, spraying the thin-layer plate with a phosphorus specific spray (Ditt-

mer and Lester, 1964) once it comes out of its developing solvent system, and then estimating in gross terms the amounts of lecithin and sphingomyelin by the visual intensities of their spots.

In our laboratory, we take a ratio of 2 or greater to predict fetal lung maturity. Gluck and his group reported a few months ago that based on the results of 302 amniocenteses from 272 pregnancies using their method of monitoring the phospholipids, not one single case of respiratory distress occurred (Gluck, Kulovich, et al, 1971). Our experience here at the Medical College of Virginia is of shorter duration. After 49 complicated pregnancies which we have monitored through 63 amniocenteses, we have not encountered any instance of respiratory distress, and among these we have had four notable cases: three of diabetes and one of Rh disease. The Rh disease patient was delivered at 33½ weeks after continuous monitoring of her lecithin-sphingomyelin ratios, and she delivered a healthy viable infant whose weight was compatible with dates. The three diabetics were delivered at 34, 35, and 36 weeks, and all delivered healthy viable infants showing no signs of respiratory distress.

Although the lecithin-sphingomyelin ratio seems to be going a long way in alleviating the frustration

of obstetricians, it should still be used with caution because the volume of data is not sufficient to show what would happen if one were to get a false positive, if one indeed ever gets false positives. The next big step that needs to be taken is a detailed study of the two major biosynthetic pathways for lecithin and a study of the characteristics and properties of the enzymes involved. If a compound could be found to induce one or both of these enzymes, this information would provide a much wanted tool in the hands of an obstetrician or pediatrician for dealing with the fetus or neonate. Such information would also be of great significance in cases of respiratory distress syndrome which are independent of obstetrical management, such as premature labor. In this instance, the physician is confronted with a situation not of his own making and has to deal with it as quickly and as best he can. At present, we are able, with the use of the described test, to predict the possibility of respiratory distress and thus have the necessary facilities such as an incubator or a respirator ready for the neonate immediately after delivery. But of even greater benefit would be the discovery of a compound drug which would induce the choline incorporation pathway. This, as I mentioned earlier, is insensitive to external insult, and once biosynthesis of lecithin has been induced by means of the pathway, the neonate itself can take over the job it is supposed to do. A further goal is the compiling of sufficient data such that a significant statistical analysis can be made concerning the accuracy of predicting the severity of respiratory distress associated with specific ranges of the lecithin/sphingomyelin ratio.

Finally, I would like to express my appreciation to our excellent housestaff who have been very patient in supplying me with samples of amniotic fluid as uncontaminated with blood as possible and also my very deep gratitude to Dr. Dunn for his constant and very enthusiastic encouragement of this work.

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