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Changes in Lamb-Lung Lipids During Gestation.* (31049)

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A considerable body of evidence has been presented recently to indicate that a substance with surface activity lines the internal surface of the lungs and that this substance helps to stabilize the alveoli during respiration(1). This surface active material, surfactant, is believed to be a lipoprotein with the activity residing in the phospholipid(2) fraction, particularly lecithin(3,4). Surfactant has been found in fetal lungs after 18 days in the mouse(5,6), 120 days in the lamb(7,8), and in human fetuses over 1,000 g in weight (9). The lungs of newborn infants dying of hyaline membrane disease usually show a reduction in surface activity(9,10) and a reduction in active phospholipid components (10).

The purpose of this report is to demonstrate both the quantitative and qualitative changes that occur in the lipid composition of saline extracts of lungs of normal fetal lambs during the last weeks of gestation. These will be related to the changes in surface activity.

Materials and methods. Fourteen fetal lambs at various gestational ages (based upon body weight) were delivered from ewes of varying genetic strains by cesarean section, taking special precautions to maintain the placental circulation intact(11). Lung specimens were obtained from each lamb by surgical biopsy and were kept frozen at —20°C until analyzed. Three grams of each lung specimen were finely minced in 50 ml of isotonic saline and then stirred for 20 minutes with a magnetic stirrer. The mixture was filtered through 2 layers of lipid-free gauze directly into a surface tension balance trough. After the surface tension measurements were made, the extract was lyophilized and used for lipid analysis. All lipid values were calculated as milligrams per 100 ml of lung saline extract.

Lipid analysis of lung saline extract. The solvents used were all American Chemical Society reagent grade and were redistilled prior to use. The filtrate of each lung saline extract was freeze-dried with a lyophilizer and was extracted in a nitrogen atmosphere, with chloroform:methanol (2:1 v/v). Total phospholipids were separated from the neutral lipids on a silicic acid column(12). The neutral lipids were eluted by chloroform and the phospholipids by methanol, and both were checked with thin layer chromatography. Lecithin, phosphatidyl ethanolamine and

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sphingomyelin were separated from the phospholipid fraction by thin layer chromatography on silica gel G using chloroform:methanol:water (60:25:25 v/v) as the solvent. About 10 mg of phospholipids were applied to the plate (20 cm × 20 cm) using about 10 g silica gel G. Standard sphingomyelin (beef brain), lecithin (egg) and phosphatidyl ethanolamine (plant) were purchased from Applied Science Laboratories, Inc., State College, Pa. These were periodically checked against purified standards obtained from Dr. James F. Mead.

The total lipids were calculated from the sum of the chloroform fraction and the methanol fraction which were evaporated to dryness in a vacuum using a flash evaporator. For determination of the individual phospholipids, one entire plate was stained with iodine vapor. A second plate was used for fatty acid analysis. Visualization of standard phospholipids and a portion of the unknown phospholipids was carried out with iodine vapor taking precautions to cover the remainder of the unknown samples. On the second plate the central lane was sprayed with 0.01 M ammonium solution of 3′,3″,5′,5″-tetrabromophenolsulfonephthalein† to identify the location of the individual phospholipids. Each band for sphingomyelin, lecithin or phosphatidylethanolamine was scraped off and extracted with chloroform methanol (1:1) five times.

The above samples were stored at −20°C after the solvents were evaporated to dryness under a stream of nitrogen. In order to check the satisfactory separation of neutral lipids from phospholipids, thin layer chromatography on silica gel G was performed using pentane:ethyl ether:glacial acetic acid (90:10:2 v/v) as the solvent. Lipid phosphorus was determined by the method of King(13).

Gas-liquid chromatography of fatty acids. The fatty acid of lecithin and neutral lipids were converted to methyl esters using boron trifluoride-methanol(14,15). Gas-liquid chromatographic analysis was carried out by means of Barber Coleman Model 10 with a 41 inch, 6 mm ID column of 12.2% w/w ethylene glycol succinate polyester on 80 to 100 mesh siliconized chromosorb at 187.0°C. The fatty acids were quantitated using peak height × retention time and known standards.

Measurement of surface tension. The surface tension of the lung saline extract from each of the lambs was measured at 22.0°C with a modified Wilhelmy balance during 80% compression and expansion of the surface film(16). The lowest value obtained with compression of the film was recorded as the minimal surface tension and the highest value obtained with expansion of the film was recorded as the maximal surface tension.

The stability index of the surface film was calculated using the formula of Clements *et al* (17) as follows:

$$\text{Stability Index (S.I.)} = \frac{2\,(\gamma\ \text{max} - \gamma\ \text{min})}{\gamma\ \text{max} + \gamma\ \text{min}}$$

where γ = surface tension.

Results. Table I contains the results of the lipid analysis and surface tension measurements obtained on each of the fetal lungs. It can be seen that all of the lipid fractions increased toward the end of gestation. The greatest increase was in the lecithin fraction which is known to be a highly surface active compound. The calculated stability index also increased in the more mature fetuses (Fig. 1).

The fatty acid composition of the lecithin fraction obtained from each of 11 fetal lungs as measured by gas chromatography is shown in Table II. The percentage of palmitic acid increased toward term, whereas the percentage of oleic acid decreased. This is shown graphically in Fig. 2.

The fatty acid composition of the neutral lipids obtained from each of the 7 fetal lungs studied by gas chromatography is shown in Table III. No consistent changes were observed between the immature and mature fetuses.

Discussion. Previous studies in fetal lambs have shown that surfactant appears between 120 to 130 days of gestation(7,8). Since the gestational age of a fetal lamb weighing 2.3 kg is approximately 120 days, these data presented herein coincide with the earlier results. In 1961, using infrared analysis, Pattle(4) found a surface-active lipoprotein in lung

† Eastman Organic Chemicals, New York.

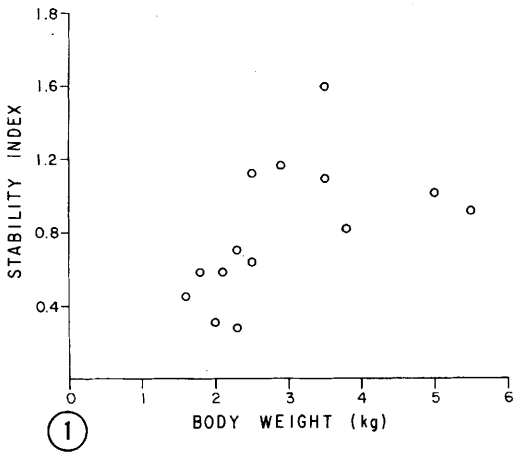
TABLE I. Lipid Analysis and Surface Activity of Fetal Lamb Lung Extracts.

Lamb No.	Body wt (kg)	Estimated fetal age (days)	Total lipids (mg %)	Neutral lipids (mg %)	Total phospho-lipids* (mg %)	Sphingo-myelin (mg %)	Lecithin (mg %)	Phosphatidyl ethano-lamine (mg %)	Stability index
Immature Group									
122	1.6	110	30.1	3.4	26.7	9.7	3.0	2.3	.46
114A	2.0	116	31.7	4.0	27.7	2.2	3.4	2.6	.31
114B	1.8	116	30.5	5.4	25.1	1.8	3.8	3.0	.58
124A	2.3	120	36.0	6.4	29.6	6.1	6.4	2.6	.71
124B	2.3	120	34.4	5.6	28.8	6.6	5.4	3.2	.28
124C	2.1	120	39.2	4.5	34.7	2.6	5.6	3.8	.58
Intermediate Group									
121	2.5	126	41.7	8.9	32.8	6.7	7.3	3.0	1.12
120A	2.5	132	48.5	8.1	40.4	5.1	6.9	3.2	.64
120B	2.9	132	41.7	8.2	33.5	8.4	7.3	5.5	1.17
Mature Group									
118A	3.5	140	46.9	6.5	40.4	15.8	15.5	7.6	1.10
118B	3.5	140	67.5	7.5	60.0	9.6	11.5	5.0	1.60
119A	5.5	150	81.6	13.8	67.8	8.6	23.2	8.2	.93
117A	3.8	150	49.0	10.4	38.6	9.5	8.8	6.0	.82
117B	5.0	150	72.5	11.4	61.1	9.2	18.3	2.8	1.05

* Methanol fraction contains contaminants as well as phospholipids.

foams with lecithin as the major component. Dipalmitoyl lecithin has been shown to be the major active constituent of lung surfactant(2,18-20). It has also been demonstrated that synthetic dipalmitoyl lecithin possesses high surface activity and produces a hysteresis curve on the surface tension balance similar to that found in lung extracts(21).

The results of the present investigation show that the milligrams of lipids per 100 ml of fetal lamb lung extract increase with body weight or gestational age. Furthermore, the percentage of palmitic acid in lecithin was found to increase with maturity, indicating a probable increase in dipalmitoyl lecithin, the major surface active material. The fatty acid composition of total lipids from lung tissue also shows an increase in palmitic acid with increase in body weight. This may indicate maturation of the lung. The quantitative and qualitative changes in lecithin may be due to the differentiation(22) of the alveolar cells as well as to the establishment of the lung circulation.



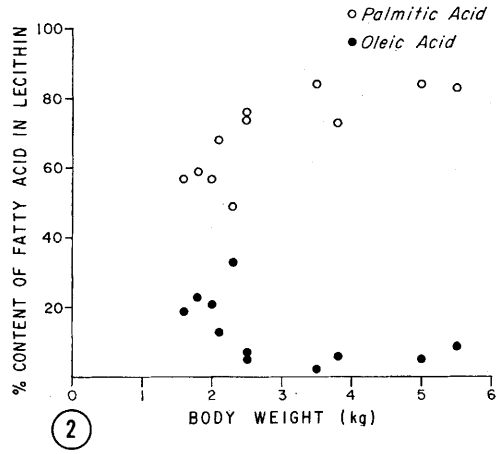


FIG. 1. Plot of stability index against fetal body weight showing increase in more mature fetuses.

FIG. 2. Plot of % content of 2 fatty acids in lecithin against fetal body weight showing increase in palmitic acid and decrease in oleic acid in more mature fetuses.

TABLE II. Fatty Acid Composition of Lecithin from Fetal Lamb Lung Extracts. (Percentage)*

Lamb No.	Body wt (kg)	$C_{12:0}$	$C_{12:1}$	$C_{14:0}$	$C_{14:1}$	(Palmitic acid) $C_{16:0}$	$C_{16:1}$	(Stearic acid) $C_{18:0}$	(Oleic acid) $C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Unsaturated fatty acid	Saturated fatty acid
122A	1.6	.2	0	1.9	.8	57.9	3.2	14.0	18.9	3.0	0	26	74
114A	2.0	tr	0	3.3	0	56.8	8.8	10.8	20.2	0	0	29	71
114B	1.8	.4	0	2.7	1.2	59.1	5.6	8.1	22.8	0	0	30	70
124A	2.3	0	0	2.2	.8	49.7	6.0	7.9	33.4	0	0	40	60
124C	2.1	tr	0	2.6	1.5	68.4	5.2	8.1	12.3	.9	.9	20	80
121A	2.5	tr	0	3.5	tr	74.9	3.7	5.4	5.8	6.8	0	16	84
120A	2.5	.9	0	4.6	1.3	76.0	2.0	9.8	5.4	0	0	9	91
118B	3.5	tr	tr	4.1	1.3	84.1	tr	5.8	2.0	2.8	0	6	94
119A	5.5	0	0	4.3	0	82.5	0	1.8	9.7	1.7	tr	11	89
117A	3.8	.6	0	2.3	2.6	73.0	tr	12.4	6.4	2.8	tr	12	88
117B	5.0	tr	tr	2.3	1.0	84.2	1.8	4.2	5.4	1.1	tr	9	91

tr = trace.
* The percentages of individual fatty acids are by weight as calculated by measuring peak height × retention time.

TABLE III. Fatty Acid Composition of Neutral Lipids from Fetal Lamb Lung Extracts. (Percentage)*

Lamb	Body wt (kg)	$C_{12:0}$	$C_{12:1}$	$C_{14:0}$	$C_{14:1}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Unsaturated fatty acids
122A	1.6	.5	0	5.4	0	45.7	0	14.6	18.6	8.3	5.9	33
114A	2.0	.2	0	1.2	.3	21.6	5.2	8.1	61.3	1.1	1.0	69
114B	1.8	.4	0	1.4	.6	43.0	5.6	8.5	30.4	8.5	1.5	47
124C	2.1	0	0	1.2	.2	25.3	4.3	8.1	59.5	1.4	0	65
120A	2.5	1.2	0	3.4	0	30.8	.6	9.7	29.7	17.1	7.5	55
119A	5.5	0	0	4.1	0	44.7	1.7	10.4	33.5	2.0	4.7	42
117B	5.0	.6	0	2.5	tr	35.0	3.0	11.5	26.4	17.5	3.4	50

tr = trace.
* The percentages of individual fatty acids are by weight as calculated by measuring peak height × retention time.

Others working with different lecithins have shown that the binding of divalent metal ions (Mg^{++}, Ca^{++}) varied with the degree of unsaturation of the fatty acids in the lecithin (23). Therefore, it is likely that the lecithin of immature lamb lung is less active to divalent metal ions than that of mature ones since the degree of unsaturation changes with maturity.

The hysteresis and low surface tension of lung extracts are reported to be dependent on the presence of subphase electrolytes(24). Thus, interaction of lecithin and electrolytes should be considered from the point of surface potential.

The results of the present experiment suggest a close relationship between surface activity and "maturity of lecithin," *i.e.*, the concentration of palmitic acid and the degree of saturation of the fatty acids.

Summary. Fourteen fetal lambs at various gestational ages were delivered by cesarean section with intact placental circulations. The saline extracts of lung sections obtained by surgical biopsy were studied for surface tension on a Wilhelmy balance, and lyophilized extracts were analyzed for lipid composition after separation on silicic acid columns and thin layer chromatography. Individual phospholipids were identified. The fatty acid composition of lecithin and neutral lipids was determined by gas chromatography. All lipid fractions increased toward the end of gestation. The greatest increase was in the lecithin fraction. The palmitic acid content in the lecithin fraction also increased toward the end

of gestation. No consistent changes were observed in the fatty acids of the neutral lipids between mature and immature fetuses. The stability index also increased toward term. These qualitative and quantitative changes observed during gestation in fetal lung extracts suggest the differentiation of the lung, which is possibly due to the maturity of alveolar cells.

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Micromethod for Determination of Specific Gravity of Tissues. (31050)

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During a study of fatty changes taking place in the liver, kidneys, and heart of a large group of fasted mice, it was noted that the specific gravity of these organs was less than those of fed mice. Hence, a rapid method for estimation of the specific gravity of these organs was needed. Since the heart samples available averaged only 25 mg, a micromethod was required. This eliminated the application of Archimedes principle used by Tsai and Lin(1) and by Saathoff(2) by which organs were weighed first in air and and then in saline solution or in water. Attaching a piece of tissue weighing only a few milligrams to a wire and determining the loss of weight in water was not practicable.

Other investigators tried flotation methods using aqueous solutions or hydrocarbon mixtures. Phillips, Van Slyke *et al*(3) adapted the flotation method to blood and plasma and utilized copper sulfate (c.s.) solutions of known specific gravity for suspending the droplets. By using a protein-coagulating medium, dispersion of material from the blood was minimized. Morales, Rathbun, and Smith(4) used this method for determining