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"Alveolar" and Whole Lung Phospholipids of Newborn Lambs* (32846)

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The mammalian lung requires a surface-active material for its alveolar stability (1–4). This material, presumably a lipoprotein complex(2,5,6) containing a highly saturated phosphatidylcholine (7–9) can be isolated from the internal surface of the lung by washing with saline.

The purpose of the present study was to determine the qualitative and quantitative nature of "alveolar" phospholipids and of whole lung tissue phospholipids of newborn lambs following the onset of breathing. Such biochemical data are a prerequisite for an understanding of the synthesis and control of secretion of pulmonary surface-active material during the neonatal period.

Materials and Methods. Fifteen newborn lambs were studied; 12 were delivered spontaneously and three were delivered by cesarean section. Each lamb was sacrificed by injection of 1% Xylocaine intracisternally, the trachea was ligated, the lamb was exsanguinated by transection of the abdominal aorta, and the lungs were removed. The right major bronchi were ligated and the left lung was separated. About 60 ml of normal saline was introduced by a syringe into the left lung through a cannulated trachea. The saline was withdrawn and returned to the lung five times. Care was taken to wash both the upper and lower lobes uniformly. The left lung was then sequentially washed five times, each with 30 ml of saline for a total of 1200 ml. Each 30-ml portion of lung wash was shaken several times and then centrifuged at 1000*g* at 5°C for 10 min to remove cells and debris. The supernatant was lyophilized.

In order to estimate the reliability of lung wash as a sampling procedure, 32 sequential saline washings of 30 ml each were carried out on the left lungs of three lambs. The lipid phosphorus was measured in the lyophilized lung wash of each 60-ml (two 30-ml washes) cell-free fraction.

Lipid analysis. The lipid was extracted from the lyophilizate with $CHCl_3$–MeOH (2:1, v/v) and washed(10). Paired samples of the extract were taken to dryness and analyzed for lipid phosphorus content(11). The lipids were fractionated into neutral lipid, nonacidic and acidic phospholipids on a DEAE[1] cellulose acetate column. The DEAE cellulose was activated according to Rouser *et al.*(12).

The lipids were eluted from the column according to the elution schema described by Gluck *et al.*(13) and the lipid phosphorus was determined on each eluent fraction. To estimate the percentage distribution of each

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[1] Diethylaminoethyl from Applied Science Laboratories.

phospholipid, components were separated by thin-layer chromatography on silica gel H (Merck) using $CHCl_3$–MeOH–H_2O (system A 95:35:4 or system B 65:25:4). The spots were made visible by exposure to iodine vapor and were then scraped off the plates. The lipid phosphorus was measured on the scrapings employing 70% perchloric acid digestion(11). Nonacidic phospholipids gave four spots (phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and an unknown) on a silica gel H coated plate in solvent system A. The *Rf* of the unknown spot was lower than that of phosphatidylethanolamine and showed no ninhydrin reactivity. A similar spot has been identified by Morgan *et al.* (9) as phosphatidyldimethylethanolamine, and intermediate in one pathway of lecithin biosynthesis. The phosphatidylcholine was separated from the nonacidic phospholipid fraction by thin-layer chromatography. Location of this phospholipid on the plate was determined by spraying with a 2′,7′-dichlorofluorescein solution (14). The lipid was eluted from the scrapings with $CHCl_3$–MeOH (1:1) and subjected to methanolysis by BF_3–methanol(15).

The positional specificity of the phosphatidylcholine molecule was determined by analyzing the fatty acids of the whole molecule and of the beta position(16). Crotalus adamanteus lyophilized venom[2] [2.0 mg in 0.2 ml of Tris buffer with 0.002 *M* $CaCl_2$ (pH 7.4)] was added to the phosphatidylcholine sample in 5 ml of diethyl ether and was incubated at room temperature for 1 hour. The fatty acid was separated by TLC, methylated(15), and analyzed for fatty acid composition. After snake venom hydrolysis, lysophosphatidylcholine was the only phospholipid evident on TLC. The suspected phosphatidyldimethylethanolamine was studied similarly. Fatty acid methyl esters were analyzed by gas liquid chromatography (Perkin-Elmer, model 881) with a diethylene glycol succinate column and hydrogen flame detector. Peak areas were calculated by multiplying peak height by peak width at half height. The total percentages of saturated fatty acids (14:0, 16:0, and 18:0) were estimated on each sample for the whole molecule and for the beta position. The values for the alpha position were calculated from these values.

[2] Ross Allen Reptile Institute, Silver Springs, Florida.

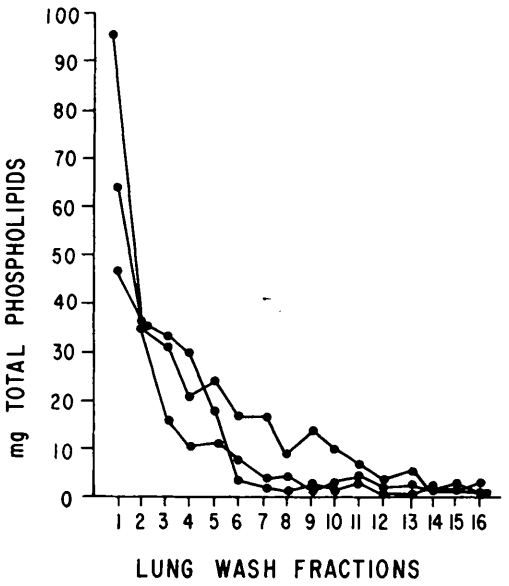

FIG. 1. Amount of phospholipid removed by sequential lung washings from the left lung of three newborn lambs. Each fraction contained 60 ml of combined wash fluid.

The minimal possible concentration of disaturated phosphatidylcholine was calculated from these data. This value was multiplied by the concentration of phosphatidylcholine to give the smallest amount of disaturated phosphatidylcholine present.

Lung tissue lipid analysis. Lipids were extracted from a portion of the right lower lobe with $CHCl_3$–MeOH (2:1, v/v) and washed (10). The analytical procedures used were the same as above. Tissue samples contiguous to those used for lipid analysis were analyzed for dry weight employing a constant temperature of 100°C for 48–72 hours. The percentage dry weight was taken as the lowest found.

Results. Sequential lung washings, when performed as described above, appear to remove virtually all of the lipid phosphorus containing material present in the interior of the lung. As shown in Fig. 1, most of the phospholipids were removed by the tenth fraction.

Table I shows the "alveolar" phospholipid

TABLE I. "Alveolar" Phospholipid Content and Composition of 15 Newborn Lambs.

Lamb no.	Age (hours)	Body wt. (kg)	Total phospho-lipids (mg)	Distribution of individual phospholipids (%)						
				PC[b]	PE	PDME	Sph	PS	PI	Others
Term fetus (8)[a]		4.6 ± 0.8	27.9 ± 4.7	86.4 ± 1.3	4.5 ± 1.2	1.8 ± 0.6	0.9 ± 0.6	2.6 ± 0.6	2.3 ± 0.7	1.5 ± 0.3
243A	1/6	4.4	48.0	89.3	3.0	1.4	0.5	3.0	2.0	6.1
244A	1/6	4.4	62.0	86.7	5.3	1.0	0.6	3.0	2.1	1.8
245A	1/6	4.4	58.0	86.0	1.4	1.0	0.9	2.8	2.9	2.0
219	1	5.0	89.4	83.0	6.0	1.0	0.4	3.0	1.2	0.2
218	2	4.0	180.0	85.7	3.4	2.0	0.8	4.0	3.0	1.1
211	18	3.4	101.3	85.0	2.5	2.0	1.0	3.5	4.0	2.5
198B	24	4.2	183.0	86.5	2.8	2.6	0.8	2.0	2.0	0.6
221	24	4.1	176.2	88.0	2.4	2.0	1.2	3.0	2.6	0.6
215	24	4.1	162.5	85.5	4.5	2.0	0.5	3.0	3.8	0.7
210	48	3.4	286.0	87.6	5.3	2.6	0.6	2.4	2.0	1.6
214	48	4.9	276.5	88.6	3.1	0.5	0.5	3.5	2.2	1.6
199	48	6.7	188.5	87.8	2.6	2.6	0.5	3.0	2.5	1.6
216	72	5.7	146.0	87.0	3.0	2.2	0.5	3.2	4.0	0.1
225	120	4.4	191.0	83.5	5.1	3.4	0.6	3.2	3.2	0.8
217	192	4.3	180.0	84.0	4.2	2.0	0.8	2.6	3.6	2.8

[a] The number in parenthesis indicates the number of animals studied.

[b] Abbrev.: PC = phosphatidylcholine; PE = phosphatidylethanolamine; PDME = phosphatidyldimethylethanolamine; Sph = sphingomyelin; PS = phosphatidylserine; PI = phosphatidylinositol; and Others = unknown + polyglycerophosphatides.

TABLE II. Percentage Total Saturated Fatty Acids, Calculated Minimum Disaturates, and Percentage Palmitic Acid of "Alveolar" Phosphatidylcholine of 15 Newborn Lambs.

Lamb no.	Age (hours)	Total saturated fatty acids			% Minimum disaturates (%)[c]	Minimum disaturated phosphatidyl-choline	Palmitic acid (%)	
		Whole	Beta	Alpha[b]			Whole	Beta
Term fetus (8)[a]		85.0 ± 2.2	77.4 ± 3.6	88.5 ± 7.4	70.3 ± 4.9	17.0 ± 3.5	82.0 ± 3.3	70.8 ± 2.8
243A	1/6	86.5	78.9	94.1	73.0	34.5	84.1	74.0
244A	1/6	83.9	79.8	88.0	67.8	36.2	82.0	72.2
245A	1/6	84.6	76.0	93.2	69.2	34.0	82.4	71.2
219	1	88.0	82.7	90.9	76.0	56.4	86.5	78.3
218	2	87.2	80.0	94.4	74.4	114.8	86.0	73.7
211	18	88.0	82.0	94.0	76.0	65.4	86.7	79.2
198B	24	87.2	85.8	88.6	74.4	117.8	85.6	82.6
221	24	86.0	80.5	81.5	62.0	93.9	85.0	79.0
215	24	92.8	88.0	97.6	85.6	129.1	92.0	86.0
210	48	88.0	86.0	90.0	76.0	190.0	84.0	79.4
214	48	90.0	89.2	90.8	80.0	196.0	85.0	82.0
199	48	91.0	88.0	94.0	82.0	135.0	86.3	80.0
216	72	90.0	85.0	95.0	80.0	101.0	86.2	79.4
225	120	90.6	87.0	95.0	83.0	133.0	87.7	83.0
217	192	86.3	82.3	90.3	73.0	110.0	84.0	77.4

[a] The number in parenthesis indicates the number of animals studied.

[b] Whole and beta position saturated fatty acids were measured, and the alpha position was calculated.

[c] Minimal disaturates = minimal percentage of saturated fatty acids at *either* alpha or beta position, which indicates concentration of alpha-saturated and beta-saturated phosphatidylcholine.

content and composition of the 15 newborn lambs. Previous values obtained on eight term nonbreathing fetuses are included for the comparison.[3] The total phospholipids increased by approximately twofold in the first 10 min as compared with the nonbreathing term fetuses. The amount continued to increase up to 48 hours of age. The percentage distribution of the individual phospholipids remained about the same during the first 8 days of life and were similar to those of term fetuses.

Table II shows the results of the analyses of the "alveolar" phosphatidylcholine obtained from the 15 newborn lambs and previous values from nonbreathing lambs for comparison. The percentage total saturated fatty acids of the whole molecule and of the beta position increased slightly after birth. Likewise, the percentage of palmitic acid of the whole molecule increased slightly. Palmitic acid at the beta position increased approximately 10% after 24 hours of age. A striking increase took place in the estimated minimal disaturated phosphatidylcholine.

[3] To be published.

Table III shows the results for the whole lung tissue phospholipids on the same 23 lambs. The total phospholipids increased after 24 hours of age. The percentage distribution of the individual phospholipids changed little with time. When the estimated minimal disaturated phosphatidylcholine present in the "alveolar" and lung tissue phospholipids are compared, the increase with time in the "alveolar" portion is striking as shown in Fig. 2.

Table IV shows the percentage saturated fatty acids of "alveolar" phosphatidyldimethylethanolamine and phosphatidylethanolamine obtained from eight of the newborn lambs compared with four term fetuses. The percentage minimal disaturated phosphatidyldimethylethanolamine was higher in the newborn lambs than the term fetuses.

Discussion. From the results presented here, it is evident that respiration of the newborn has a profound effect upon the liberation of surface-active material onto the alveolar space. The amount of "alveolar" phospholipid increased twofold in the first 24 hours and

TABLE III. Lung Tissue Phospholipids of 15 Newborn Lambs.

Age (hours)	Total phospholipids (mg/gm of dry wt.)	Distribution of individual phospholipids (%)						Phosphatidylcholine	
		PC[c]	PE	Sph	PS	PI	Others	Minimum disaturates (%)[b]	Minimum disaturated phosphatidylcholine (mg/gm of dry wt.)
Term fetus (8)[a]	121.6 ± 3.5	60.4 ± 0.8	12.3 ± 1.3	16.0 ± 1.3	5.0 ± 0.8	3.8 ± 0.7	2.4 ± 0.4	39.4 ± 7.4	30.5 ± 5.2
1/6	126.0	60.4	12.0	15.6		12.0		44.0	33.5
1/6	136.2	58.0	11.0	15.2		15.7		43.0	34.0
1/6	123.0	57.3	12.3	15.0		15.4		38.8	27.4
1	126.0	60.0	14.6	17.5		10.0		38.0	28.0
2	130.0	60.0	12.7	16.5		10.8		36.0	28.0
18	130.6	58.3	14.9	18.3		7.6		42.0	32.0
24	139.0	60.5	13.0	16.0		10.5		47.6	38.6
24	141.0	69.0	12.0	14.0		12.0		42.4	35.2
24	142.0	70.2	9.2	11.0		10.9		48.0	34.0
48	180.0	72.7	11.0	10.0		6.3		50.0	65.4
48	145.0	70.5	9.8	10.7		9.9		42.4	43.3
48	162.0	70.4	10.0	11.0		8.6		46.0	52.6
72	146.5	58.6	20.7	13.7		7.2		48.0	44.0
120	146.7	67.7	14.3	10.7		7.3		32.4	32.2
192	140.0	62.1	12.4	13.0		12.5		51.0	44.3

[a] The number in parenthesis indicates the number of animals studied.

[b] Minimal disaturates = minimal percentage of saturated fatty acids at either alpha or beta position, which indicates concentration of alpha-saturated and beta-saturated phosphatidylcholine.

[c] Abbrev.: PC = phosphatidylcholine; PE = phosphatidylethanolamine; Sph = sphingomyelin; PS = phosphatidylserine; PI = phosphatidylinositol; and Others = unknown + polyglycerophosphatides.

TABLE IV. The Percentage Saturated Fatty Acids Contained in Phosphatidyldimethylethanolamine and Phosphatidylethanolamine Obtained from Lung Washings from Newborn Lambs of Different Ages as Compared with Those of Nonbreathing Term Fetal Lambs.

		Age (hours)							
	Term fetus (4)[a]	1/6		1	2	24		120	192
Phosphatidyldimethylethanolamine (%)									
Whole	68.7 ± 7.1	63.4	70.0	71.0	72.2	88.0	83.8	80.5	88.0
Beta	64.0 ± 7.1	55.6	65.0	62.5	65.0	84.8	75.6	78.6	82.0
Alpha[b]	73.4 ± 7.5	71.2	76.0	79.5	79.4	91.2	92.0	82.4	94.0
Minimum disaturates[c]	37.2 ±14.2	26.8	40.0	42.0	44.4	76.0	67.6	61.0	76.0
Phosphatidylethanolamine (%)									
Whole	44.3 ± 5.8	59.0	54.8	—	58.0	56.0	62.0	—	—

[a] The number in parenthesis indicates the number of animals studied.

[b] Total and beta position saturated fatty acids were measured and the alpha position was calculated.

[c] Minimum disaturates = minimal percentage of saturated fatty acids at either alpha or beta position, which indicates concentration of alpha-saturated and beta-saturated phosphatidyldimethylethanolamine.

eightfold in the second day of life. The "alveolar" phospholipid composition of the newborn lamb is similar to that of the term fetus reported previously (Table I), but differs from that of whole lung phospholipids. Furthermore, the "alveolar" phosphatidylcholine of the newborn contains more disaturated phophatidylcholine than that of whole lung. The "alveolar" disaturated phosphatidylcholine represents the major portion of dipalmitoyl phosphatidylcholine (Table II).

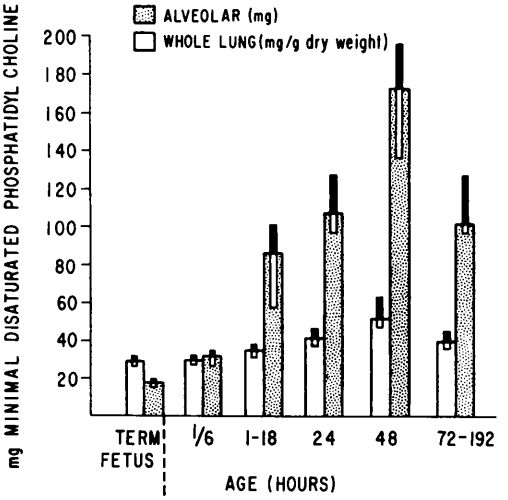

FIG. 2. "Alveolar" and lung tissue minimal disaturated phosphatidylcholine content of term fetal newborn lambs. Mean and range of values.

An increase in the "alveolar" disaturated phosphatidylcholine content in the newborn lamb may occur as the result of either mechanical or metabolic factors. With the onset of respiration, air inflation establishes an alveolar–air interface. The surface-active material seeks the surface and lines the alveoli. Respiration likely produces a continuous liberation of the surface-active material from the alveolar lining cells.

Electron-microscopic observations suggest that a greater amount of osmiophilic lining layer is present in the alveoli of newborn lambs than in fetal animals(17). The results of the present study support these observations and the concept that the osmiophilic lining layer is the surface-active material(17–19). Since the increase in the "alveolar" disaturated phosphatidylcholine in the newborn lamb that has breathed for several hours is not accompanied by an appreciable change in whole lung disaturated phosphatidylcholine, mechanical factors may play a major role in the increase observed during this period.

The concentration of whole lung disaturated phosphatidylcholine increases from the first to second day of life. Concomitantly, the "alveolar" disaturated phosphatidylcholine reaches the maximum level on the second day

of life. The "alveolar" phosphatidylcholine on the second day of life contains more disaturated fatty acids than that of term fetuses. The increase in the phosphatidylcholine saturated fatty acids occurs primarily at the beta position (Table II) and is due to an increase in palmitic acid at the beta position which might be by exchange. These findings suggest that further synthesis and accumulation of surface-active material take place during this period of life. With the onset of breathing, pulmonary blood flow increases by tenfold and this provides more substrate for lung metabolism. Although there is no direct evidence that the synthesis of surface-active material is oxygen dependent, the lung is known to be an active site of a high oxygen consumption(20) and of phospholipid synthesis(21). The synthesis of phospholipid is energy-dependent, being influenced by the availability of oxygen(22).

The larger amounts of both "alveolar" and whole lung tissue disaturated phosphatidylcholine in the newborn than the adult animal (16,23) may represent a reserve of surface-active material. This may be related to the metabolic and functional stability of mature lung and also linked to evidence that the full-term newborn is resistant to the development of respiratory distress(24,25). Morgan *et al.* (9) have found phosphatidyldimethylethanolamine in the "alveolar" phospholipids of adult dogs and suggest that surface-active phosphatidylcholine is synthesized from phosphatidylethanolamine by methylation. This phospholipid was present in the "alveolar" phospholipids of mature fetuses and newborn lambs (Table IV). The percentage minimal disaturated phosphatidyldimethylethanolamine in newborn lambs is higher than in mature fetuses. This suggests the significance of the methylation pathway as a selective process in which disaturated phosphatidylcholine, a principal component of the surface-active material, is formed.

Summary. Fifteen newborn lambs ranging in age from 10 min to 192 hours were studied. The qualitative and quantitative nature of "alveolar" phospholipids and whole lung tissue phospholipids were determined and compared with data previously obtained on term fetuses. Sequential lung washings removed practically all of the surface-active material from the internal surface of the lung. The "alveolar" phospholipids increased twofold in 10 min after the onset of respiration, fourfold in the first 24 hours and eightfold in the second day of life. The "alveolar" phospholipid composition of the newborn is similar to the term fetus, but differs from whole lung tissue phospholipids. The percentage minimal disaturates of the phosphatidylcholine of the newborn is slightly higher than that of the term fetus. "Alveolar" disaturated phosphatidylcholine represents the major portion of dipalmitoyl phosphatidylcholine. Phosphatidyldimethylethanolamine was isolated from "alveolar" phospholipids. This phospholipid contains a higher concentration of disaturated phosphatidyldimethylethanolamine in the newborn than in the fetus. It is concluded that respiration of the newborn has a profound effect both mechanically and metabolically upon the liberation and elaboration of surface-active material.

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Effects of Hypothalamic Lesions on Incidence and Growth of Mammary Tumors in Carcinogen-Treated Rats* (32847)

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The onset and growth of mammary adenocarcinomas in female rats given carcinogens apparently require both anterior pituitary and ovarian hormones. Thus rats ovariectomized or hypophysectomized prior to carcinogen treatment develop few or no mammary tumors (1), and ovariectomy or hypophysectomy after mammary tumor development can result in tumor regression (2). Further evidence of the importance of anterior pituitary hormones, particularly prolactin, is provided by experiments showing that single or multiple pituitary transplants can increase the incidence of mammary tumors in mice (3). Prolonged injections of prolactin into intact female mice also resulted in mammary tumors (4), as did transplantation of pituitary "mammotropic" tumors in mice (5) and rats (6).

Placement of lesions in the median eminence (ME) of the tuber cinereum results in enhanced release of prolactin and in marked reduction in release of all other anterior pituitary hormones (7). It was of interest therefore, to study the effects of ME lesions on the induction and growth of mammary tumors in intact and ovariectomized rats treated with a carcinogen.

Materials and Methods. Placement of ME lesions before DMBA. Seventy-five virgin female Sprague-Dawley rats (Holtzman Co., Madison, Wisc.), all uniform in body weight were divided randomly into four groups. At 56 days of age all animals were given a single intravenous injection of 1 ml of a lipid emulsion containing 5 mg of 7,12-dimethylbenzanthracene (DMBA).[3] Additional treatments were as follows: group 1, intact controls, no treatment; group 2, intact, bilateral lesions placed in the ME at 50 days of age; group 3, ovariectomized at 64 days of age and no further treatment; group 4, placement of bilateral lesions in the ME at 50 days of age and ovariectomized at 64 days of age. After carcinogen treatment all animals were examined once weekly for a period of 6 months for development of mammary tumors. All ME lesions were produced by passing a direct current of 2 mA for 7 sec through epoxylite coated steel electrodes prepared from insect pins. The ME was located with the aid of a

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