Quantification of surfactant pool sizes in rabbit lung during perinatal development

M. Oulton,[1] M. Fraser, M. Dolphin, R. Yoon, and G. Faulkner

Departments of Obstetrics/Gynecology, Physiology/Biophysics, and of Microbiology, Dalhousie University, Halifax, Nova Scotia, B3H 4H7, Canada

Abstract Methods are presented for the quantitative isolation of surfactants from fetal and newborn rabbit alveolar lavage returns and post-lavaged lung tissue homogenates. The phospholipid content of both fractions progressively increased between 27 days gestation and term (31 days). The tissue-stored fraction increased approximately 16-fold (from 0.48 ± 0.13 to 7.83 ± 0.86 mg/g dry lung) and the alveolar fraction more than 30-fold (from 0.08 ± 0.02 to 2.69 ± 0.52 mg/g dry lung). Developmental changes in phospholipid composition were also observed. Tissue-stored surfactant was prepared using differential and density gradient centrifugation. Alveolar surfactant was isolated during fetal development as a high-speed pellet following a one-step differential centrifugation. There was little change in the phospholipid content of fetal alveolar lavage supernatant (range 0.12 ± 0.04 to 0.28 ± 0.09 mg/g dry lung). By the first postnatal day the phospholipid content of both lavage fractions significantly increased (pellet, 7.51 ± 1.79; supernatant, 4.01 ± 1.36 mg/g dry lung) and both were identified as surfactant. This increase in alveolar surfactant was accompanied by an approximately twofold decrease (to 3.81 ± 1.1 mg/g dry lung) in the tissue-stored fraction. These data provide a quantitative profile of surfactant accumulation and secretion in developing rabbit lung. — **Oulton, M., M. Fraser, M. Dolphin, R. Yoon, and G. Faulkner.** Quantification of surfactant pool sizes in rabbit lung during perinatal development. *J. Lipid Res.* 1986. **27:** 602–612.

Supplementary key words lamellar inclusion bodies • alveolar lavage • phospholipids • fetal lung development

Lung surfactant is a phospholipid-rich material that lines the mammalian lung and prevents atelectasis by reducing the surface tension at the air–alveolar interface (1). Disaturated species of phosphatidylcholine (2) and phosphatidylglycerol (3, 4) are the major phospholipid constituents responsible for this surface tension-reducing property. Surfactant is synthesized in the alveolar Type II cell and stored in intracellular lamellar inclusion bodies prior to release to the alveoli (5).

In the developing fetus, lung surfactant production is initiated in the last trimester of gestation and progressively increases toward term (6). Deficient surfactant at birth predisposes the newborn to the development of the respiratory distress syndrome (RDS), a disease characterized by progressive atelectasis and possible death to the infant (7, 8). Although many hormones, including glucocorticoids and other agents, have been shown to accelerate surfactant production (9), the mechanism by which this process is controlled during fetal development is not well understood.

Much of our current knowledge of the development of the fetal lung surfactant system has been provided by morphological (10) and physiological (11) studies. Little information is available on the quantitative aspects of this process, i.e., the levels of intracellular stores and the extent of release to the airways. Such information is of fundamental value in understanding how this process is controlled.

In recent years several methods involving differential and density gradient centrifugation have been developed for isolating surfactant material from alveolar lavage returns and/or lung tissue homogenates (12). However, with the exception of one study performed by Katyal, Estes, and Lombardi (13) on developing fetal rat lung, most studies of surfactant isolated by these methods have been qualitative and were largely performed on fractions obtained from adult lung. The purpose of the present study was to devise a method for isolating surfactant from alveolar lavage returns and post-lavaged lung tissue in fetal and newborn rabbits and to determine the phospholipid content and composition of these fractions during normal perinatal development. By separately isolating surfactant from the two lung compartments, the developmental pattern of intracellular surfactant accumulation (fraction isolated from post-lavaged lung tissue) and the extent of release to the alveoli (fraction isolated from alveolar lavage) can be determined.

Abbreviations: RDS, respiratory distress syndrome; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; SM, sphingomyelin; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine.

[1]Address correspondence and reprint requests to: Dr. M. Oulton, Sir Charles Tupper Medical Building, Room 5G4, Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia, B3H 4H7, Canada.

MATERIALS AND METHODS

New Zealand white rabbits, whose time of conception was known to within 1 hour, were purchased from Rieman's Fur Ranch, St. Agatha, Ontario. The does were anesthetized with intravenous sodium pentobarbital, and the fetuses were delivered by cesarean section and immediately killed by intraperitoneal injection of sodium pentobarbital. Some does were allowed to deliver spontaneously at term and the newborns were killed as above 1–2 days later.

Fetal and newborn lungs were lavaged in situ with a total of seven lavages (0.75–1.5 ml of 0.15 M NaCl) and the lavage returns obtained for each experiment (from one to three litters per experiment) were pooled for fractionation as described below. Adult lungs were similarly lavaged with seven 25-ml aliquots of 0.15 M NaCl. The residual lung tissue obtained from each experiment was pooled, finely minced, and portions were removed and dried to constant weight at 120°C. Surfactant was isolated from the remaining tissue by a modification of the procedure of Frosolono et al. (14) as follows.

All steps in the isolation procedure were performed at 0°C. A 10% homogenate (w/v) in 0.01 M Tris buffer, containing 0.145 M NaCl and 0.001 M EDTA (pH 7.4) was prepared using a Polytron Homogenizer (Brinkman Instruments, Rexdale, Ontario) at a speed setting of 5.0 for 10–30 sec (in 10-sec bursts). The homogenate was centrifuged for 5 min at 140 *g* and the resultant supernatant for 30 min at 10,000 *g*. The 10,000 *g* pellet was suspended in the above-mentioned Tris buffer and 2.0-ml aliquots were layered over discontinuous density gradients consisting of 5.0 ml each of 0.68 M and 0.25 M sucrose (prepared in the Tris buffer); the gradients were centrifuged for 60 min at 65,000 *g*. A crude surfactant fraction which banded at the interface between the sucrose layers was removed, recovered as a pellet by centrifugation for 30 min at 10,000 *g*, resuspended in Tris buffer, and subjected to a second density gradient centrifugation as described above. The final surfactant fraction was pelleted as above, washed once in Tris buffer, and finally suspended in 0.15 M NaCl for lipid extraction.

The recovery of surfactant from tissue homogenate was estimated by preparing a radiolabeled surfactant fraction and carrying it through the isolation procedure. To perform this experiment, the doe at 30 days gestation was given an intravenous injection of 1 mCi of [9,10-^{3}H(N)]palmitic acid (23.5 Ci/mmol, New England Nuclear, Lachine, Quebec) which had previously been converted to the sodium salt and stabilized with BSA as described by Jobe, Kirkpatrick, and Gluck (15). After 24 hr, ^{3}H-labeled surfactant was isolated from the fetal lung tissue as described above and equal aliquots of this preparation (containing 5.63×10^4 dpm) were added in duplicate to *a*) fetal lung tissue homogenate obtained on the same day from fetuses of an untreated doe and *b*) an equivalent volume of homogenizing medium. Each mixture was then carried through the entire isolation procedure and aliquots were removed at each step for determination of radioactivity in an LKB liquid scintillation counter.

The homogeneity of the final preparation was assessed in one experiment in which duplicate aliquots of the banded material obtained at 31 days gestation were subjected to density gradient centrifugation by both upward flotation according to Duck-Chong (16) and downward sedimentation, according to Frosolono et al. (14) through linear sucrose gradients ranging from 0.9 to 0.2 M sucrose. All sucrose solutions were made up in the above-mentioned Tris buffer; their concentrations were checked by refractometry (Carl Zeiss, Germany) at 25°C and converted to density at 0°C (17). Each gradient was centrifuged for 120 min at 65,000 *g* and the rotor was brought to rest without braking. After noting their positions in the gradients, the banded fractions were removed for phospholipid analysis.

To estimate the recovery of phospholipids by alveolar lavage, fetal lungs were lavaged 12 times and the phospholipid content of each consecutive lavage return was determined. To isolate surfactant from alveolar lavage returns, the lavage fluid was centrifuged for 30 min at 10,000 *g*. In preliminary studies the 10,000 *g* pellet obtained from fetal (28–31 days gestation) alveolar lavage returns was subjected to density gradient centrifugation as described above. However, as this step resulted in no further purification of the surfactant fraction (both with respect to phospholipid composition and electron microscopic appearance), it was not employed for routine analyses. These analyses were, therefore, performed on the 10,000 *g* pellets. For comparative purposes, analyses were separately performed on the 10,000 *g* supernatant fractions. To maintain procedural consistency, newborn and adult preparations were handled in the same manner.

Lipid extraction and phospholipid analysis were performed as described elsewhere (18). Methyl esters of the fatty acyl chains of phosphatidylcholine and phosphatidylglycerol were obtained by transesterification with 10% BF_3 in methanol (w/v) and extraction with petroleum ether (19). The methyl esters were analyzed by gas–liquid chromatography as described by Cook (20) except that a 10% Silar 9 CP Series 3000-3 Hi-Eff phase (Applied Science Laboratories, Inc., State College, PA) was run isothermally at 205°C.

Aliquots were taken from fetal (30–31 days gestation) and adult lung tissue homogenates for preparation of mitochondria and microsomes as described by Harding et al. (21), and plasma membranes according to Maeda, Balakrishnan, and Mehdi (22). Succinate dehydrogenase (EC 1.3.99.1) was measured as described by Pennington (23), NADPH-cytochrome c reductase (EC 1.6.2.4) by the method of Omura and Takesue (24), and 5'-nucleotidase according to Avruch and Wallach (25). Protein was measured according to Lowry et al. (26).

Surface tension measurements were performed at room temperature (23°C) with a modified Langmuir-Wihelmy surface balance (Cahn Instruments/Division of Ventron, Cerriotos, CA) essentially as described by Levine and Johnson (27). The maximum surface area was 44.6 cm^2 and the minimum was 11.2 cm^2. The compression and expansion of the surface took place in 11.6 sec. Alveolar lavage supernatants were examined directly and, to maintain consistency in analysis, lavage pellets as well as density gradient fractions obtained from the post-lavaged lung tissue were suspended in 0.15 M NaCl for testing. By this method, a minimum phospholipid concentration of 30 μg/ml was required to achieve a minimum surface tension (γmin) of less than 15 dynes/cm.

Electron microscopy was performed as previously described (18).

RESULTS

Following centrifugation of the first density gradient of the post-lavaged lung tissue homogenate (**Fig. 1**), one band of material, at the interface between the sucrose layers, and one rather large pellet were consistently observed. On density gradient centrifugation of the banded material, the bulk of this fraction again banded at the interface between the sucrose layers, but a small pellet was always present. The phospholipid compositions of the pellet, as well as the supernatant fractions obtained after both density gradient centrifugations, were similar but were different from that of the banded material (**Table 1**). In particular, the banded material contained a higher proportion of PI and PC and lower proportions of SM and PE than either the pellet or the supernatant fractions. The second density gradient resulted in the removal of approximately 15% of the total phospholipid from the banded material. Further density gradient centrifugations resulted in no further removal of material from the banded fraction. The banded material obtained in the second density gradient at each gestational age exhibited surface activity (γmin:28-day fetus, 4.2 ± 1.4 dynes/cm for three determinations; newborn, 1.7 ± 0.6 dynes/cm for three determinations) and was taken as the final surfactant preparation. No other fractions obtained in the isolation procedure exhibited surface activity (results not shown).

The purity of the final surfactant preparation was assessed by mitochondrial (succinate dehydrogenase), microsomal (NADPH-cytochrome c reductase), and plasma membrane (5'-nucleotidase) markers (**Table 2**) and by electron microscopy (**Fig. 2**). The results of the marker enzyme analyses (Table 2) indicate less than 2% mitochondrial and less than 5% microsomal and plasma membrane contamination for both fetal and adult surfactant preparations. Electron microscopy of fetal preparations (Fig. 2) revealed the presence of mainly intact lamellar bodies with minimal contamination by extraneous membrane components. When subjected to centrifugation on linear sucrose gradients, the surfactant material, whether floated upwards or pelleted downwards, was recovered as a single, distinct band in the region corresponding to 0.4 M sucrose (d 1.055 g/ml).

In assessing the recovery of a ^{3}H-labeled surfactant preparation carried through the isolation procedure, it was found that, when added to the homogenizing medium, at least 90% of the radioactivity was recovered in the final surfactant fraction, while an addition to the tissue homogenate recovery was reduced to approximately 85%. The extra 5% loss occurred in the initial centrifugation step but could be recovered as a banded fraction by density gradient centrifugation of the 140 *g* pellets. The remaining 10% loss was rather evenly distributed over the other fractions obtained in the isolation procedure. In a separate set of experiments we found that, on density gradient centrifugation of the 140 *g* pellet, we consistently recovered a surfactant band that constituted less than 5% of the total recoverable surfactant phospholipids. For routine analysis we did not attempt to recover this small amount of material, so the results presented are those obtained on analysis of the banded material recovered from the 10,000 *g* pellet only.

The phospholipid content of surfactant isolated from post-lavaged lung tissue, progressively and almost linearly, increased between 27 and 31 days (term) gestation (**Fig. 3**). The overall increase during this time period was 16-fold. On the first postnatal day, the phospholipid content of this fraction was greatly reduced but increased toward the adult level by the second postnatal day. The phospholipid composition of the isolated surfactant fractions progressively changed during development (**Table 3**). At each gestational age PC represented the major phospholipid and, as a percent of the total lipid phosphorus, progressively increased up to the first postnatal day. With respect to the acidic phospholipids, as a percent of total lipid phosphorus, PS progressively decreased during development; PG, which represents the major acidic phospholipid in adult surfactant, first appeared in measurable quantities in fetal surfactant at 28–29 days gestation, after which time it progressively increased toward the adult value and PI decreased concomitantly with the increase in PG. The adult values for these latter two acidic phospholipids were not as yet attained by the second postnatal day. General overall decreases in both SM and PE were also found.

Developmental changes were found in the fatty acid composition of both PC and PG obtained from the surfactants isolated from post-lavaged lung tissue (**Table 4**). The major fatty acid of both phospholipids was 16:0 which

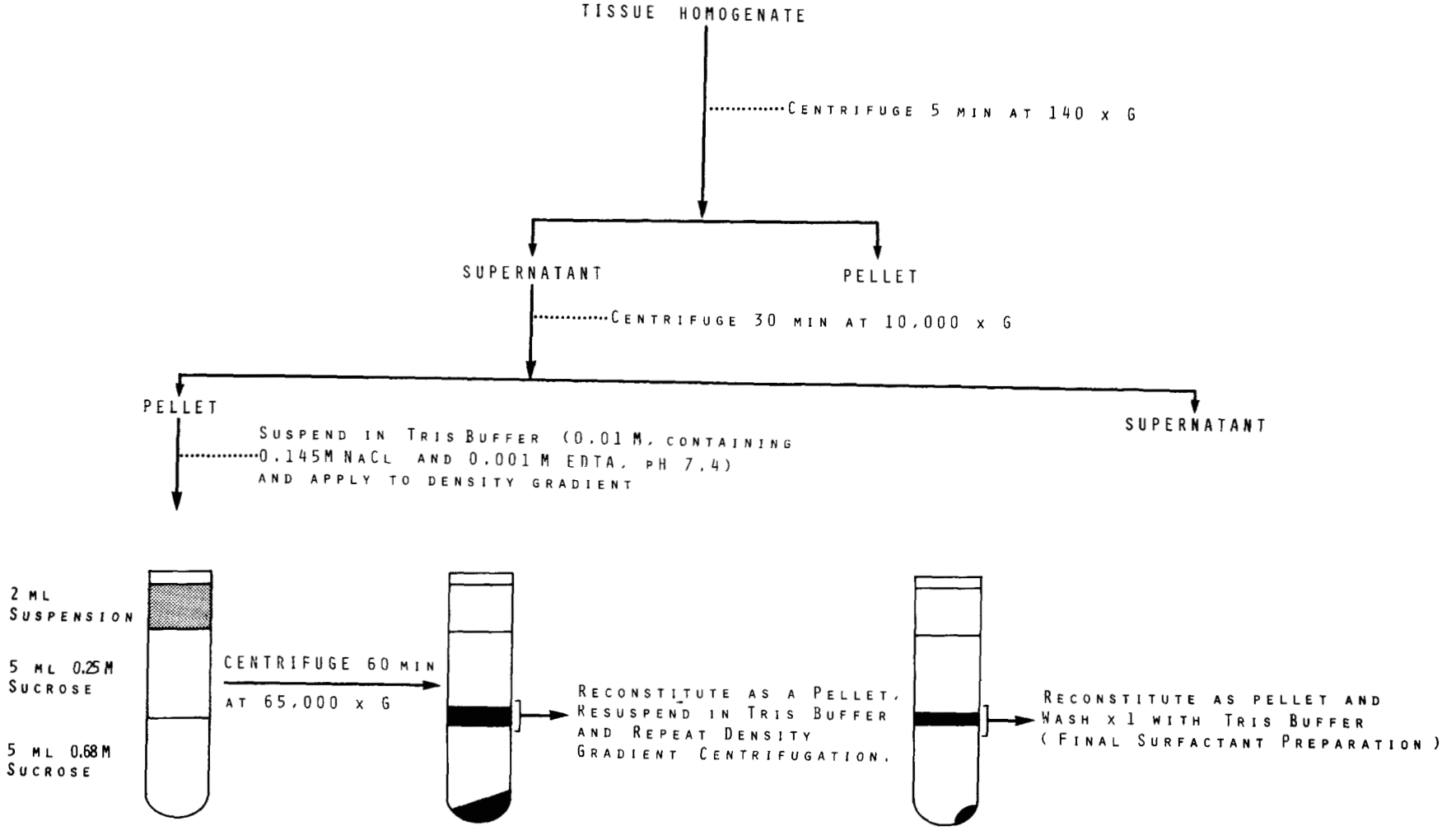

Fig. 1. Outline of procedure for isolating surfactant from post-lavaged lung tissue. Sucrose solutions were prepared in Tris buffer (0.01 M, containing 0.145 M NaCl and 0.001 M EDTA, pH 7.4).

TABLE 1. Phospholipid composition of density gradient fractions isolated from post-lavaged lung tissue

Fraction	n	PS	PI	SM	PC	PG	PE	X	LPC
					% of total lipid phosphorus				
Surfactant band	8	2.7 ± 0.4[a]	11.8 ± 0.4	0.8 ± 0.2	77.4 ± 0.9	0.3 ± 0.2	6.7 ± 0.5	0.3 ± 0.2	N.D.[b]
Supernatants I–III	3	5.9 ± 1.5	7.9 ± 0.5	10.2 ± 2.8	53.3 ± 3.2	0.3 ± 0.5	20.9 ± 2.2	N.D.	1.5 ± 0.5
Pellet	3	7.6 ± 0.5	6.3 ± 1.5	8.5 ± 0.5	49.6 ± 2.2	1.3 ± 1.0	25.1 ± 3.9	0.7 ± 0.9	0.8 ± 0.3

Abbreviations used are: PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; X, unidentified phospholipid; LPC, lysophosphatidylcholine.

[a]Each value represents the mean ± 1 SD for the number of determinations shown for each fraction. Analyses were performed on fractions obtained following the second density gradient centrifugation from fetal rabbit lung at 29 days gestation.

[b]Not detectable.

decreased slightly at birth but then increased in the adult. At each gestational age, 16:0 comprised a greater proportion of PC (at least 60%) than PG (approximately 50%) fatty acids. The second most abundant PC fatty acid in the fetal preparations was 16:1, while in the adult it was 18:1. The second most abundant PG fatty acid was 18:1 which declined somewhat at birth but then increased in the adult, concomitant with a decrease in 16:1 and 18:2.

With respect to the recovery of phospholipids by alveolar lavage, it was found that greater than 90% (91.6 ± 1.1% for a total of five experiments at 31 days gestation) of the phospholipid present in a total of twelve successive lavages could be recovered by the first seven. Although only a few studies of this type were performed on earlier gestation fetuses, a similar pattern of recovery was found. For routine analysis, a total of seven lavages was used for each gestational age examined but smaller volumes were used for the earlier gestation fetuses. In each experiment, greater than 90% of the total NaCl solution instilled was recovered in the lavage returns.

Phospholipid analyses were performed separately on the 10,000 *g* supernatant and pellet fractions of the alveolar lavage returns. As indicated in **Fig. 4,** during fetal development the bulk of the phospholipid was recovered in the pellet fraction and the phospholipid content of this fraction progressively increased from 27 to 31 days gestation. There was an overall 33-fold increase during this time period with an approximately 9-fold increase from 27 to 29 days gestation and a further 3.6-fold increase from 29 to 31 days gestation. The progressive increase in phospholipid content of this fraction coincides with that found in the surfactant fractions isolated from the post-lavaged lung tissue. In contrast, the phospholipid content of the alveolar lavage supernatant fraction remained relatively constant during fetal development. One day following birth, the phospholipid content of both alveolar lavage fractions markedly increased (coincident with the significant decrease found in surfactant isolated from the residual tissue) and continued to increase toward adult levels.

In comparing the phospholipid compositions of the pellet and supernatant fractions of alveolar lavage returns (**Table 5**), significant differences were noted during fetal development. These differences were particularly evident in the earlier gestation fetuses (i.e., at 27 and 28 days gestation). In general, the supernatant fraction contained relatively less PC and PI and relatively more SM than the pellet fraction. Also LPC, which was not present in the pellet fraction, represented a relatively high proportion (9–10%) of the phospholipids present in the supernatant fraction. The supernatant composition gradually changed so that by 31 days gestation it appeared to approach that of the pellet fraction. In the newborn, no significant differences were noted in the phospholipid compositions of the two alveolar lavage fractions and the composition of both was similar to that of the surfactant fraction obtained

TABLE 2. Distribution of marker enzymes in subcellular fractions of fetal and adult rabbit lung

	Succinate Dehydrogenase		NADPH: Cytochrome c Reductase		5'-Nucleotidase	
	Fetus	Adult	Fetus	Adult	Fetus	Adult
			nmol/min per mg protein			
Mitochondria	43.5[a]	12.1				
Microsomes			19.6	29.3		
Plasma membranes					55.5	91.4
Surfactant	0.6	0.2	0.7	1.2	1.9	4.5

[a]Each value represents the mean of from two to four determinations. Fetal values were obtained from preparations isolated at 30–31 days gestation.

Fig. 2. Electron micrograph of surfactant isolated from post-lavaged lung tissue at 28 days gestation demonstrating the presence of mainly intact lamellar bodies with minimal contamination of extraneous membrane components; × 52,500.

from the post-lavaged lung tissue at the same age of development.

During fetal development, particularly after 28 days gestation, the pellet fraction of the alveolar lavage returns bore a closer resemblance in phospholipid composition to the surfactant fraction isolated from post-lavaged lung tissue at the same gestational age than did the supernatant fraction. At 28 days gestation, the pellet fraction contained slightly more SM than the surfactant isolated from post-lavaged lung tissue but thereafter no significant differences in composition were observed in the surfactant fractions isolated from the two compartments.

Fatty acid analysis (**Table 6**) revealed significant differences between lavage pellet and supernatant PC, but not PG, at each of the gestational ages examined. As with the overall distribution of phospholipids, the fatty acid composition of the fetal pellet but not the supernatant PC was almost identical to that of the surfactant isolated from the post-lavaged lung tissue. This was particularly evident in the 28-day gestation fetus.

During fetal development, the lavage pellet, but not the supernatant, exhibited surface activity (γmin:pellet, 5.8 ± 3.2 dynes/cm; supernatant, 24.8 ± 2.3 dynes/cm, each for four determinations from 29–31 days gestation). In the newborn, both pellet and supernatant fractions exhibited surface activity (γmin:pellet, 2.0 ± 0.9 dynes/cm; supernatant, 8.3 ± 2.1 dynes/cm, each for three determinations on 1- to 2-day-old newborns). We did not test lavage pellets from adults; however, the supernatant fraction exhibited surface activity (γmin:5.3 ± 0.9 dynes/cm for three determinations). Electron microscopy was performed on alveolar lavage pellets obtined from fetal lung only and revealed the presence of mainly multilamellated structures (**Fig. 5**).

DISCUSSION

Several methods involving differential and density gradient centrifugation have been developed for isolating surfactant fractions from alveolar lavage returns and/or lung tissue homogenates (12). In the present report we describe methods for the sequential isolation of surfactants from the two lung compartments and the quantitative as

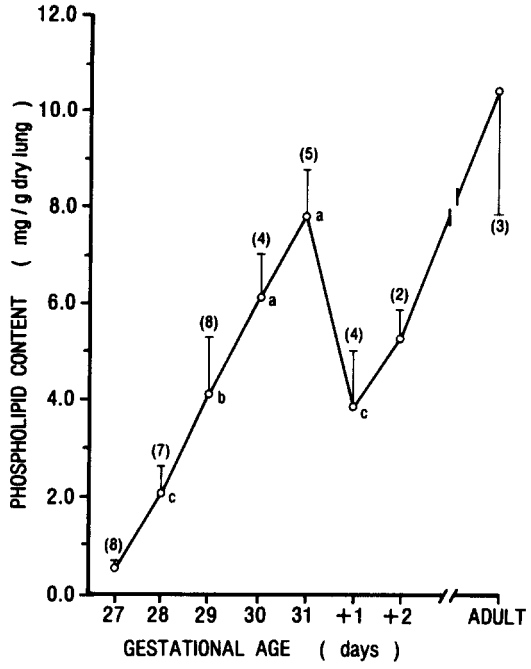

Fig. 3. Phospholipid content of surfactant isolated from post-lavaged lung tissue during perinatal development. Each value represents the mean ± 1 SD for the number of determinations shown in parentheses. The values designated as a, b, and c are significantly different from those obtained at the previous gestational age at $P < 0.02$, 0.01, and 0.001, respectively.

well as the qualitative aspects of these fractions during perinatal development in the rabbit.

With respect to surfactant isolated from the post-lavaged lung tissue, our results indicate the recovery of a reasonably pure lamellar body preparation. Although our fetal preparations, in contrast to those isolated by other investigators (4, 13), demonstrated both mitochondrial and microsomal contamination, we expect that since our surfactant preparations contained at least eight times as much phospholipid as protein (by weight) that the low levels of contamination that we found would contribute minimally to the phospholipid analysis of the surfactant fraction.

Our demonstration of a less than 15% loss of lamellar bodies during the entire isolation procedure is in agreement with that reported for adult dog (14) and rat (13) but contrasts with the approximately 80% loss reported for adult rabbit (28). The large losses encountered in the latter study likely occurred in the initial stages of the isolation procedure which involved a 10-min centrifugation at 900 *g* followed by filtration through cheesecloth. We found, for example, that an initial centrifugation for 5 min at 600 *g* resulted in a 25–40% loss of surfactant material and that cheesecloth filtration could result in even further losses. This loss was reduced to approximately 5% by employing the much lower 140 *g* spin as an initial step to remove cellular debris.

In several experiments we recovered the surfactant band from the 140 *g* pellet and, on the phospholipid analysis, found that this fraction consistently represented less than 5% of the surfactant material recovered from the 10,000 *g* pellet. It therefore appeared acceptable to exclude this step (which is very time-consuming) from our routine procedure. No other pellet fractions obtained during the isolation procedure yielded a surfactant band when subjected to density gradient centrifugation and no fraction other than the surfactant band demonstrated surface activity. Also, on examination of the tissue homogenate by electron microscopy, there was no evidence of unbroken alveolar Type II cells and the quantity of surfactant material isolated at each gestational age was highly reproducible and independent of the starting tissue weight. We therefore feel that the method we have adopted is satisfactory for the quantitative isolation of tissue-stored surfactant from rabbit lung during perinatal development. While many investigators have used the Potter-Elvehjem homogenizer to prepare lung tissue for surfactant isolation (12), our results indicate that the Polytron homogenizer, at least under the conditions described in this report, can also be used for this step. The necessity of a second density gradient step to purify the surfactant has not been reported previously and could result from the low *g* force which we use to remove cells

TABLE 3. Phospholipid composition of surfactant isolated from post-lavaged lung tissue during perinatal development

Gestational Age	n	PS	PI	SM	PC	PG	PE	X	LPC
days					*% of total lipid phosphorus*				
27	8	5.7 ± 1.1[a]	9.3 ± 0.8	3.5 ± 1.2	66.7 ± 3.0	N.D.	14.0 ± 1.5	0.7 ± 0.7	N.D.
28	7	3.8 ± 0.7	11.2 ± 0.6	1.4 ± 0.7	74.2 ± 2.0	0.3 ± 0.2	9.1 ± 1.0	0.1 ± 0.1	N.D.
29	8	2.7 ± 0.4	11.8 ± 0.4	0.8 ± 0.2	77.4 ± 0.9	0.3 ± 0.2	6.7 ± 0.5	0.3 ± 0.2	N.D.
30	4	2.4 ± 0.3	11.7 ± 0.3	0.3 ± 0.4	78.1 ± 0.9	1.2 ± 0.4	6.1 ± 0.3	0.2 ± 0.3	N.D.
31	5	2.1 ± 0.3	10.9 ± 1.8	0.9 ± 0.5	78.4 ± 2.2	1.5 ± 0.9	5.6 ± 0.7	0.5 ± 0.4	N.D.
+1	4	1.3 ± 0.2	7.2 ± 0.7	0.5 ± 0.3	82.3 ± 2.0	3.4 ± 1.0	4.9 ± 0.4	0.3 ± 0.5	N.D.
+2	2	1.5 ± 0.4	6.2 ± 0.6	0.9 ± 0.1	82.5 ± 0.7	3.6 ± 0.1	4.2 ± 0.4	1.2 ± 0.1	N.D.
Adult	3	1.1 ± 0.4	3.8 ± 0.3	0.3 ± 0.2	82.0 ± 1.3	6.8 ± 0.2	4.3 ± 0.2	1.5 ± 0.2	0.2 ± 0.2

[a]Each value represents the mean ± 1 SD for the number of determinations shown.
Abbreviations used are as indicated in Table 1.

TABLE 4. Fatty acid composition of phosphatidylcholine and phosphatidylglycerol in surfactant isolated from post-lavaged lung tissue during perinatal development

Fatty Acid Methyl Ester	Phosphatidylcholine				Phosphatidylglycerol		
	28 (3)[a]	31 (3)	+1 (3)	Adult (2)	31 (2)	+1 (3)	Adult (2)
14:0	4.1 ± 0.9[b]	7.8 ± 0.3	6.9 ± 0.5	3.9 ± 0.0	2.0 ± 1.2	2.3 ± 0.5	0.7 ± 0.0
15:0	1.3 ± 0.2	1.8 ± 0.1	1.3 ± 0.1	0.9 ± 0.0	0.4 ± 0.2	1.5 ± 1.4	0.3 ± 0.1
16:0	66.4 ± 2.2	65.4 ± 1.5	61.9 ± 1.2	67.9 ± 1.1	50.3 ± 5.0	47.2 ± 5.6	55.3 ± 3.3
16:1	12.4 ± 1.5	13.3 ± 1.2	15.6 ± 0.4	6.7 ± 0.8	10.6 ± 0.4	11.3 ± 3.2	5.6 ± 0.5
18:0	2.5 ± 0.6	0.8 ± 0.1	1.0 ± 0.2	2.3 ± 0.4	5.8 ± 0.4	5.7 ± 1.8	6.2 ± 1.7
18:1	7.4 ± 1.5	4.4 ± 0.3	6.1 ± 0.6	10.3 ± 1.3	19.5 ± 4.0	13.7 ± 4.2	26.4 ± 0.6
18:2	4.0 ± 0.3	3.5 ± 1.4	5.4 ± 0.3	5.6 ± 0.8	8.7 ± 0.8	6.5 ± 1.3	3.8 ± 2.2
18:3	0.1 ± 0.1	0.2 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	2.1 ± 2.1	N.D.
Other[c]	1.9 ± 0.4	2.6 ± 1.1	1.3 ± 0.3	2.4 ± 0.3	2.3 ± 0.4	9.8 ± 8.8	1.7 ± 0.4

[a]Gestational age in days; number of determinations at each age in parentheses.
[b]The data are expressed as a percentage by weight (± 1 SD) of the total fatty acid methyl esters. Fatty acid methyl esters were identified by reference to known methyl ester standards; N.D., not detectable.
[c]Contains arachidonic acid (20:4) as well as other unidentified long-chain constituents.

and nuclei. The phospholipid composition of our adult preparations, as well as the compositional changes observed during fetal and newborn development, are in agreement with those reported by others (4, 13, 29–31). The steady increase in surfactant phospholipid content from 27 days to term and the dramatic decrease immediately following birth concurs with reported morphological studies (32). In view of the large amounts of tissue required in this study to obtain a surfactant fraction at 27 days gestation and also the failure of others (33) to isolate surfactant at 26 days gestation, we did not attempt to extend our studies to earlier developmental stages.

With respect to alveolar lavage, while there has up to now been little investigation into isolating surfactant fractions during fetal development, previous studies have indicated that high-speed centrifugation does not necessarily sediment all of the surfactant in alveolar lavage returns obtained from either adult (31, 34) or newborn (35) lung. Studies by Magoon et al. (31) in the adult rabbit suggest that this effect is due to the presence of different ultrastructural arrangements of alveolar surfactant (tubular myelin figures, multi- or unilamellar membrane components) with different sedimentation properties and that the lighter components may be structures that have been partially processed for re-uptake and reassembly into lamellar bodies by the alveolar Type II cell. This process has also been described in newborn rabbits (36). In the present study, although we did not examine the ultrastructure of our newborn and adult lavage fractions, based on their overall compositions and surface activities, we feel that both the high speed pellet and supernatant fractions can be regarded as surfactant. The overall compositions we report for these fractions are similar to surfactants isolated by others (4, 29, 31, 37, 38).

In the fetus, however, our results strongly indicate that the alveolar lavage pellet, which consists primarily of multi-lamellated vesicles typical of fetal surfactant (18, 39, 40) is representative of secreted surfactant, while the phospholipids of the lavage supernatant fraction appear to be of a non-surfactant origin. However, as the supernatant phospholipid composition gradually approaches that of the pellet towards term, it is possible that at term there may be small but indeed negligible (in view of the relative phospholipid contents of pellet vs. supernatant) quantities of surfactant material remaining in the supernatant fraction.

Prolonged centrifugation (at *g* forces as high as 1000 *g*

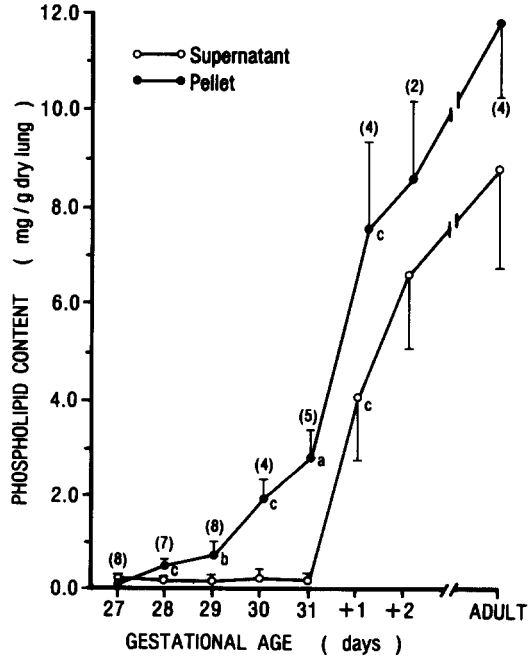

Fig. 4. Phospholipid content of alveolar lavage fractions during perinatal development. Each value represents the mean ± 1 SD for the number of determinations shown in parentheses. Statistical analyses are as described for Fig. 3. Lavage supernatant (○), lavage pellet (●).

TABLE 5. Phospholipid composition of alveolar lavage fractions determined at different stages of perinatal development

Fraction	n	PS	PI	SM	PC	PG	PE	X	LPC
					% of total lipid phosphorus				
27 days									
Supernatant	8	4.5 ± 2.4	4.5 ± 2.5[a]	31.6 ± 2.3[a]	35.8 ± 2.8[a]	N.D.	12.5 ± 2.0[a]	1.3 ± 1.8	10.0 ± 3.0[a]
Pellet	8	5.0 ± 4.0	8.7 ± 2.8	17.7 ± 3.8[b]	51.9 ± 3.4[b]	N.D.	16.3 ± 2.3[b]	0.5 ± 0.5	N.D.
28 days									
Supernatant	7	3.3 ± 1.2	7.4 ± 1.3[a]	27.2 ± 2.9[a]	42.7 ± 3.8[a]	N.D.	10.0 ± 0.7[a]	0.3 ± 0.7	9.3 ± 2.3[a]
Pellet	7	3.4 ± 0.3	11.1 ± 0.3	4.0 ± 0.9[b]	73.7 ± 2.6	0.5 ± 0.3	6.9 ± 2.9	0.5 ± 0.5	N.D.
31 days									
Supernatant	5	3.0 ± 2.2	10.6 ± 1.2	5.0 ± 2.3	70.0 ± 6.6	0.7 ± 1.7	5.8 ± 1.3	1.9 ± 2.0	3.0 ± 2.1[a]
Pellet	5	1.8 ± 0.3	11.4 ± 1.5	0.5 ± 0.5	79.1 ± 1.4	1.9 ± 1.1	5.0 ± 0.3	0.5 ± 0.3	N.D.
+1 day									
Supernatant	4	1.4 ± 0.3	7.9 ± 2.3	0.9 ± 0.6	80.7 ± 3.4	3.6 ± 0.9	4.1 ± 0.9	1.0 ± 1.1	N.D.
Pellet	4	1.9 ± 0.5	7.2 ± 0.5	1.5 ± 1.2	80.6 ± 1.4	4.0 ± 0.8	4.4 ± 0.3	0.6 ± 0.7	N.D.

Each value represents the mean ± 1 SD for the number of determinations shown (n). Abbreviations used are as indicated in Table 1.
[a]Significantly different from pellet fraction at the same gestational age ($P < 0.02$).
[b]Significantly different from surfactant fraction isolated from residual tissue at the same gestational age.

for 15 min) has been employed by other investigators (30, 37) to prepare fetal alveolar lavage returns for phospholipid analysis. While it is recognized that this provides a useful means for removing the cellular fraction from adult lavage, our study demonstrates the virtual absence of cells from our fetal preparations. This, taken together with the large loss of phospholipid encountered even with a prior centrifugation for 5 min at 140 *g*, suggests that not only is such a preparatory step unnecessary but that it should be avoided. These procedural losses, which were also encountered on density gradient centrifugation of the 10,000 *g* pellet, were most evident when the lowest levels of surfactant were present, i.e., at the earliest gestational ages, and partially explain why previously reported phospholipid compositions (30, 37) obtained for fetal rabbit lavage samples following prolonged centrifugation more closely resemble our supernatant than our pellet fractions.

In the rabbit fetus, the time period from 27 to 29 days gestation represents a critical stage in development marking the transition from a functionally immature to a functionally mature lung (41). It would, therefore, be expected that dramatic changes in alveolar surfactant levels would occur during this time period. While this is not evident when a prior preparatory centrifugation step is employed (37, present study), it does become evident when this step is avoided. Based on the phospholipid content and overall composition of our 10,000 *g* pellet fractions, our studies suggest that at 27 days gestation, negligible, if any, surfactant has been released to the alveoli, that identifiable surfactant first appeared at 28 days gestation, and that the quantity nearly doubled by

TABLE 6. Fatty acid composition of phosphatidylcholine and phosphatidylglycerol in alveolar lavage fractions during perinatal development

	Phosphatidylcholine						Phosphatidylglycerol	
	28-Day Fetus		31-Day Fetus		Newborn		Newborn	
Fatty Acid Methyl Ester	Supernatant (3)[a]	Pellet (3)	Supernatant (3)	Pellet (2)	Supernatant (3)	Pellet (3)	Supernatant (3)	Pellet (2)
14:0	3.8 ± 1.0	3.0 ± 0.5	5.9 ± 0.6	5.6 ± 1.4	5.5 ± 0.3[b]	7.0 ± 0.6	1.7 ± 0.3	1.8 ± 0.5
15:0	1.2 ± 0.3	1.1 ± 0.2	0.9 ± 0.3	1.3 ± 0.4	1.0 ± 0.2[b]	1.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.4
16:0	55.6 ± 4.8[b]	70.4 ± 2.7	65.4 ± 2.5	69.0 ± 0.6	67.1 ± 0.4	64.1 ± 1.7[c]	50.7 ± 3.2	53.8 ± 3.1
16:1	6.9 ± 0.7[b]	12.5 ± 1.6	12.3 ± 0.9	13.3 ± 0.4	15.0 ± 0.6	14.2 ± 0.3[c]	9.3 ± 0.7	11.9 ± 2.3
18:0	7.3 ± 1.8[b]	2.0 ± 0.6	1.2 ± 0.5	0.6 ± 0.2	0.6 ± 0.0	1.0 ± 0.1	4.9 ± 0.6	4.0 ± 0.8
18:1	11.4 ± 2.6[b]	6.5 ± 0.3	4.5 ± 0.0	4.7 ± 0.5	5.6 ± 0.3	5.7 ± 0.1	15.1 ± 0.5	12.8 ± 2.5
18:2	5.5 ± 1.2	3.0 ± 1.5	4.6 ± 0.8	4.5 ± 0.5	4.6 ± 0.5	4.4 ± 1.0	5.4 ± 0.1	4.7 ± 0.9
18:3	0.2 ± 0.4	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.2	0.4 ± 0.1	0.7 ± 0.1	1.1 ± 0.8
Other	8.3 ± 1.7[b]	1.4 ± 0.4	5.2 ± 1.7[b]	0.9 ± 0.3	0.3 ± 0.1[b]	1.1 ± 0.1	11.8 ± 3.9	9.5 ± 0.9

Expression of data and identification of fatty acid methyl esters are as indicated in Table 4.
[a]Number of determinations of each fraction in parentheses.
[b]Significantly different from pellet fraction at the same gestational age ($P < 0.05$).
[c]Significantly different from surfactant fraction isolated from post-lavage lung tissue at the same gestational age ($P < 0.05$).

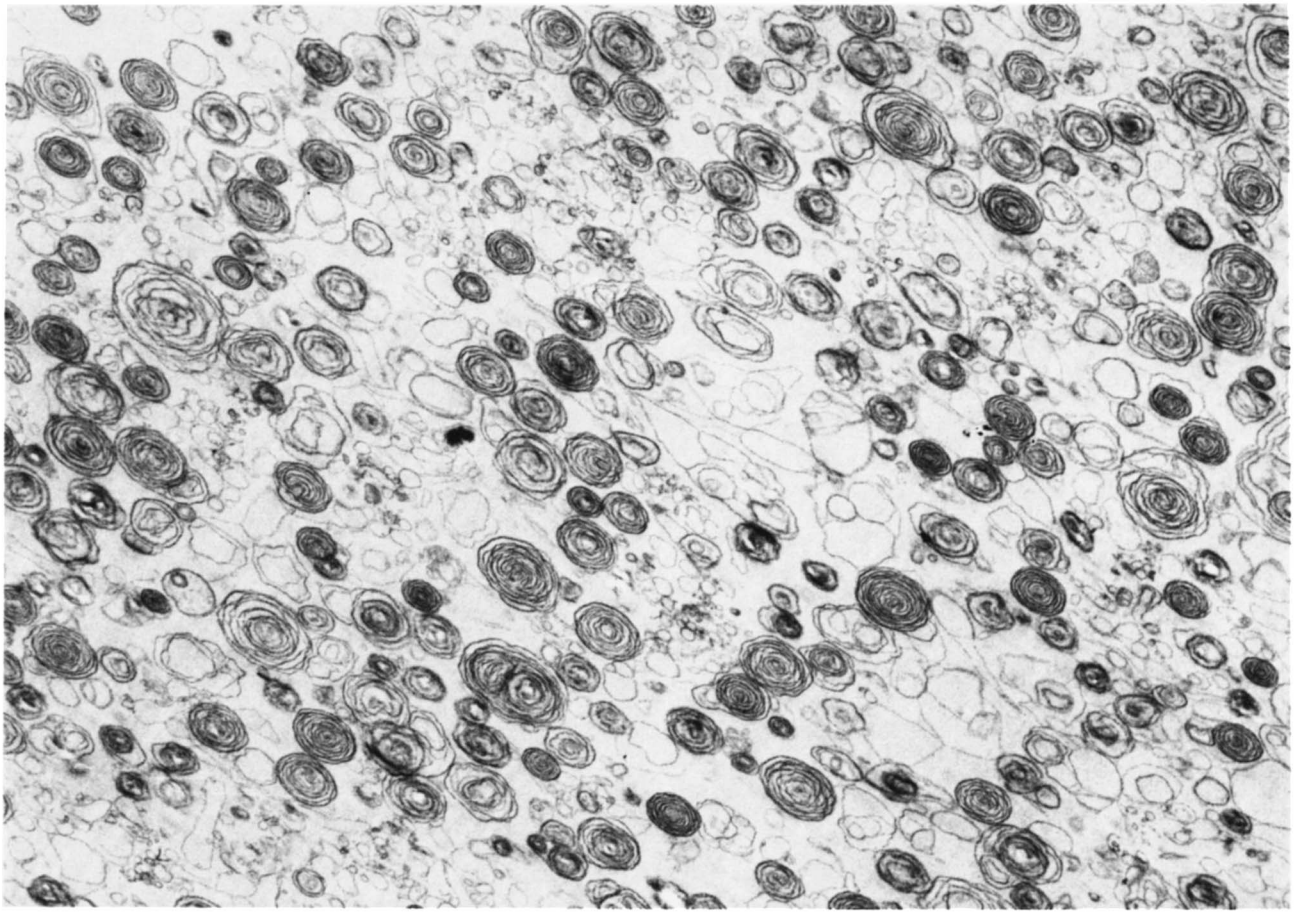

Fig. 5. Electron micrograph of fetal (31 days gestation) alveolar lavage pellet illustrating presence of mainly multilamellated structures; × 8,000.

29 days gestation. These results are in agreement with electron microscopic studies (10, 27) which indicate the first appearance of surfactant in the alveoli at 27–28 days gestation. We thus conclude that, in studies involving both the qualitative and quantitative aspects of surfactant secretion during fetal development, the most feasible approach to isolating the surfactant fraction from alveolar lavage returns is to use the one-step centrifugation procedure described in this report.

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REFERENCES

1. Goerke, J. 1974. Lung surfactant. *Biochim. Biophys. Acta.* **344:** 241–261.
2. King, R. J., and J. A. Clements. 1972. Surface active materials from dog lung. II. Composition and physiological correlations. *Am. J. Physiol.* **223:** 715–726.
3. Rooney, S. A., P. M. Canavan, and E. K. Motoyama. 1974. The identification of phosphatidylglycerol in the rat, rabbit, monkey and human lung. *Biochim. Biophys. Acta.* **360:** 56–67.
4. Hallman, M., and L. Gluck. 1976. Phosphatidylglycerol in lung surfactant. III. Possible modifier of surfactant function. *J. Lipid Res.* **17:** 257–262.
5. Askin, F. B., and C. Kuhn. 1971. The cellular origin of pulmonary surfactant. *Lab. Invest.* **25:** 260–268.
6. Blackburn, W. R. 1973. Hormonal influences in fetal lung development. *In* Respiratory Distress Syndrome. C. A. Villee, D. B. Villee, and J. Zuckerman, editors. Academic Press, New York. 271–293.
7. Adams, F. H., T. Fujiwara, G. Emmanouilides, and A. Scudder. 1965. Surface properties and lipids from lungs of infants with hyaline membrane disease. *J. Pediatr.* **66:** 357–364.
8. Avery, M. E., and J. Mead. 1959. Surface properties in relation to atelectasis and hyaline membrane disease. *Am. J. Dis. Child.* **97:** 517–523.
9. Ballard, P. L. 1982. Hormonal aspects of fetal lung development. *In* Lung Development: Biological and Clinical Perspectives. II. Neonatal Respiratory Distress. P. M. Farrell, editor. Academic Press, New York. 205–253.
10. Kikkawa, Y., M. Kaibara, E. K. Motoyama, M. M. Orzalesi, and C. D. Cook. 1971. Morphological development of fetal rabbit lung and its acceleration with cortisol. *Am. J. Pathol.* **64:** 423–442.

11. Taeusch, H. W., Jr., I. Wyszogradski, N. S. Wang, and M. E. Avery. 1974. Pulmonary pressure-volume relationships in premature fetal and newborn rabbits. *J. Appl. Physiol.* **37:** 809–813.
12. Frosolono, M. F. 1982. Bronchoalveolar lavage fluid and subcellular surfactant fractions in lung development. *In* Lung Development: Biological and Clinical Perspectives. I. Biochemistry and Physiology. P. M. Farrell, editor. Academic Press, New York. 111–134.
13. Katyal, S. L., L. W. Estes, and B. Lombardi. 1977. Method for isolation of surfactant from homogenates and lavages of lungs of adult, newborn and fetal rats. *Lab. Invest.* **36:** 585–592.
14. Frosolono, M. F., B. L. Charms, R. Pawlowski, and S. Slivka. 1970. Isolation, characterization, and surface chemistry of a surface-active fraction from dog lung. *J. Lipid Res.* **11:** 439–457.
15. Jobe, A., E. Kirkpatrick, and L. Gluck. 1978. Lecithin appearance and apparent biologic half-life in term newborn rabbit lung. *Pediatr. Res.* **12:** 669–675.
16. Duck-Chong, C. G. 1978. The isolation of lamellar bodies and their membranous content from rat lung, lamb tracheal fluid and human amniotic fluid. *Life Sci.* **22:** 2025–2030.
17. Hames, B. D. 1984. Choice of conditions for density gradient centrifugation. *In* Centrifugation: A Practical Approach. 2nd ed. D. Rickwood, editor. IRL Press, Oxford. 45–93.
18. Oulton, M., T. R. Martin, G. T. Faulkner, D. Stinson, and J. P. Johnson. 1980. Developmental study of a lamellar body fraction isolated from human amniotic fluid. *Pediatr. Res.* **14:** 722–728.
19. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride–methanol. *J. Lipid Res.* **5:** 600–608.
20. Cook, H. W. 1981. Metabolism of triacylglycerol in developing rat brain. *Neurochem. Res.* **6:** 1217–1229.
21. Harding, P. G. R., F. Chan, P. G. Casola, G. F. Fellows, T. Wong, and F. Passmayer. 1983. Subcellular distribution of the enzymes related to phospholipid synthesis in developing rat lung. *Biochim. Biophys. Acta.* **750:** 373–382.
22. Maeda, T., K. Balakrishnan, and S. W. Mehdi. 1983. A simple and rapid method for the preparation of plasma membranes. *Biochim. Biophys. Acta.* **731:** 115–120.
23. Pennington, R. J. 1961. Biochemistry of dystrophic muscle: mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase. *Biochem. J.* **80:** 649–654.
24. Omura, T., and S. Takesue. 1970. A new method for simultaneous purification of cytochrome b_5 and NADPH-cytochrome c reductase from rat liver microsomes. *J. Biochem.* **67:** 249–257.
25. Avruch, J., and D. F. H. Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochem. Biophys. Acta.* **233:** 334–347.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
27. Levine, B. E., and R. P. Johnson. 1964. Surface activity of saline extracts from inflated and degassed normal lungs. *J. Appl. Physiol.* **19:** 333–335.
28. Hallman, M., B. L. Epstein, and L. Gluck. 1981. Analysis of labelling and clearance of lung surfactant phospholipids in rabbit: evidence for bidirectional flux between lamellar bodies and alveolar lavage. *J. Clin. Invest.* **68:** 742–751.
29. Sanders, R. L. 1982. The composition of pulmonary surfactant. *In* Lung Development: Biological and Clinical Perspectives. I. Biochemistry and Physiology. P. M. Farrell, editor. Academic Press, New York. 193–210.
30. Hallman, M., and L. Gluck. 1980. Formation of acidic phospholipids in rabbit lung during perinatal development. *Pediatr. Res.* **14:** 1250–1259.
31. Magoon, M. W., J. R. Wright, A. Baritussio, M. C. Williams, J. Goerke, B. J. Benson, R. L. Hamilton, and J. A. Clements. 1983. Subfractions of lung surfactant: implications for metabolism and surface activity. *Biochim. Biophys. Acta.* **750:** 18–31.
32. Kikkawa, Y., E. K. Motoyama, and L. Gluck. 1968. Study of the lungs of fetal and newborn rabbits: morphological biochemical and surface physical development. *Am. J. Pathol.* **52:** 177–209.
33. Gutcher, G. R., R. D. Zackman, and F. H. C. Tsao. 1979. Analysis of subcellular phosphatidylcholine in developing rabbit lung. *Lipids.* **14:** 25–29.
34. Massaro, M., L. Clerch, and G. D. Massaro. 1981. Surfactant aggregation in rat lungs: influence of temperature and ventilation. *J. Appl. Physiol.* **51:** 646–653.
35. Moxley, M. A., R. D. Harlow, and R. V. Kotas. 1982. Sedimentation characteristics of newborn rabbit surfactant. *Federation Proc.* **41:** 1602 (Abstract).
36. Jacobs, H., A. Jobe, M. Ikegami, and D. Conoway. 1983. The significance of re-utilization of surfactant phosphatidylcholine. *J. Biol. Chem.* **258:** 4156–4165.
37. Rooney, S. A., S. T. Wai-Lee, L. Gobran, and E. K. Motoyama. 1976. Phospholipid content, composition and biosynthesis during fetal development in the rabbit. *Biochim. Biophys. Acta.* **431:** 447–458.
38. Jobe, A., E. Kirkpatrick, and L. Gluck. 1978. Labelling of phospholipids in the surfactant and subcellular fractions of rabbit lung. *J. Biol. Chem.* **253:** 3810–3816.
39. Williams, M. C. 1977. Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. *J. Cell Biol.* **72:** 260–277.
40. Hook, G. E. R., L. B. Gilmore, E. G. Tombropoulos, and S. E. Fabro. 1978. Fetal lung lamellar bodies in human amniotic fluid. *Am. Rev. Respir. Dis.* **117:** 541–550.
41. Kotas, R. V., and M. E. Avery. 1971. Accelerated appearance of pulmonary surfactant in the fetal rabbit. *J. Appl. Physiol.* **30:** 358–361.