Fatty acids of pulmonary surfactant phosphatidylcholine from fetal rabbit lung tissue in culture. Biosynthesis of n–10 monoenoic fatty acids

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Abstract We have previously reported that fetal rabbit lung tissue in organ culture produces a lamellar body material (pulmonary surfactant) with a lower percentage of disaturated phosphatidylcholine than is typically found in rabbit lung in vivo (Longmuir, K. J., C. Resele-Tiden, and L. Sykes. 1985. *Biochim. Biophys. Acta.* **833:** 135–143). This investigation was conducted to identify all fatty acids present in the lamellar body phosphatidylcholine, and to determine whether the low level of disaturated phosphatidylcholine is due to excessive unsaturated fatty acid at position *sn*-1, *sn*-2, or both. Fetal rabbit lung tissue, 23 days gestation, was maintained in culture for 7 days in defined (serum-free) medium. Phospholipids were labeled in culture with [1-^{14}C]acetate or [U-^{14}C]glycerol (to follow de novo fatty acid biosynthesis), or with [1-^{14}C]palmitic acid (to follow incorporation of exogenously supplied fatty acid). Radiolabeled fatty acid methyl esters obtained from lamellar body phosphatidylcholine were first separated by reverse-phase thin-layer chromatography (TLC) into two fractions of *1*) 14:0 + 16:1 and *2*) 16:0 + 18:1. Complete separation of the individual saturated and monoenoic fatty acids was achieved by silver nitrate TLC of the two fractions. Monoenoic fatty acid double bond position was determined by permanganate–periodate oxidation followed by HPLC of the carboxylic acid phenacyl esters. Lamellar body phosphatidylcholine contained four monoenoic fatty acids: *1*) palmitoleic acid, 16:1 *cis*-9; *2*) oleic acid, 18:1 *cis*-9; *3*) *cis*-vaccenic acid, 18:1 *cis*-11; and *4*) 6-hexadecenoic acid, 16:1 *cis*-6. In addition, 8-octadecenoic acid, 18:1 *cis*-8, was found in the fatty acids of the tissue homogenate. The abnormally low disaturated phosphatidylcholine content in lamellar body material was the result of abnormally high levels of monoenoic fatty acid (principally 16:1 *cis*-9) found at position *sn*-2. Position *sn*-1 contained normal levels of saturated fatty acid. The biosynthesis of the unusual n–10 fatty acids was observed from the start of culture throughout the entire 7-day culture period, and was observed in incubations of tissue slices of day 23 fetal rabbit lung. This is the first report of the biosynthesis of n–10 fatty acids (16:1 *cis*-6 and 18:1 *cis*-8) in a mammalian tissue other than skin, where these fatty acids are found in the secretory product (sebum) of sebaceous glands. — **Longmuir, K. J., C. Resele-Tiden, and M. E. Rossi.** Fatty acids of pulmonary surfactant phosphatidylcholine from fetal rabbit lung tissue in culture. Biosynthesis of n–10 monoenoic fatty acids. *J. Lipid Res.* 1988. **29:** 1065–1077.

Supplementary key words fatty acid desaturation • fatty acid chain elongation • palmitoleic acid • 6-hexadecenoic acid

Pulmonary surfactant is a lipid–protein complex formed by the type II alveolar epithelial cell in the mammalian lung (1). Following synthesis of the lipid and protein components, the product accumulates in cytoplasmic structures called lamellar bodies. The contents of the lamellar body (lamellar body material) are secreted onto the alveolar surface to become the pulmonary surfactant. The principal lipid of surfactant is disaturated phosphatidylcholine, which is mostly responsible for lowering alveolar surface tension (2).

In vitro culture of small tissue pieces of mammalian fetal lung (organ culture) has been accepted as a valid model system for studying type II epithelial cell development, and for studying the regulation of the biosynthesis of lamellar body material (3–12). In culture in serum-free medium, the columnar epithelium of lung tissue differentiates to type II cells, which produce lamellar body material and secrete it into fluid-filled spaces within the tissue pieces (3, 4). Addition of hormones to the medium is not required for differentiation and lamellar body formation to occur (3, 6). Instead, a variety of pharmacologic agents, such as corticosteroids, thyroid hormones, theophylline, and estrogen, will augment the synthesis and accumulation of lamellar body material by several fold (3, 5–12).

In a series of investigations, we have reported that the morphology and phospholipid profile of lamellar body material, isolated from fetal rabbit lung in culture, is virtually identical to lamellar body material obtained from lung in vivo (13–15). Also, it has been recently reported that the major 29–36 kDa glycoprotein specifically associated with rabbit lung surfactant is expressed in the organ culture system (16). However, the

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; FAME, fatty acid methyl ester.

lamellar body material from culture contains a lower percentage of disaturated phosphatidylcholine than is found in fetal lung in vivo, due to the abundance of palmitoleic acid, 16:1 *cis*-9, in phosphatidylcholine (15). This unusual fatty acid profile is a reproducible finding in this culture system, making it a useful, comparative laboratory model for understanding which metabolic steps are important for the regulation of disaturated phosphatidylcholine biosynthesis.

In this communication, we report the results of radiolabeling experiments that were performed to analyze the fatty acid content of lamellar body phosphatidylcholine from fetal rabbit lung in culture. We determined that the low disaturated phosphatidylcholine content was because of excessive unsaturated fatty acid at position *sn*-2 and not at position *sn*-1. Also, we determined that this lung tissue carries out the biosynthesis of two unusual monoenoic fatty acids, 16:1 *cis*-6 and 18:1 *cis*-8. Previously, this biosynthetic activity has been reported to take place only in the sebaceous glands of skin epithelium (17–22).

MATERIALS AND METHODS

Materials

Tissue culture supplies were obtained from Falcon. Medium and antibiotics were obtained from Gibco. Radioactive materials were purchased from ICN ([1-^{14}C]palmitic acid and [U-^{14}C]glycerol) and Amersham ([1-^{14}C]acetate). Supplies and reagents for electron microscopy were purchased from Ladd Research Industries. Silica gel 60 and RP-18 TLC plates were from E. Merck. Solvents (distilled in glass) were purchased from American Burdick and Jackson. Diethyl ether contained 2% ethanol preservative instead of butylated hydroxytoluene. Perchloric acid (70%), double distilled in Vycor, was purchased from G. Frederick Smith and used for lipid phosphorus analyses. Boron trifluoride (12% in methanol) was purchased from Supelco. Phospholipase A_2 (porcine pancreas) was purchased from Sigma. Platinum(IV) oxide catalyst was purchased from Aldrich. Reagents for permanganate–periodate oxidation, for preparation of phenacyl esters, and for HPLC are described separately (23). Other reagents were analytical grade.

Culture of lung tissue

Tissue pieces of fetal rabbit lung, 23 days gestation, were prepared for culture as described previously (14, 15). Tissue was cultured in Waymouth's MB 752/1 medium, with antibiotics, without serum, and without added hormones (15). Cultures were maintained for 7 days. Radiolabeling was carried out over the entire 7-day culture period with medium containing [1-^{14}C]palmitic acid (10 μM, 6.4 μCi/μmol), [U-^{14}C]glycerol (8.3 μM, 16 μCi/μmol), or [1-^{14}C]acetate (4.6 μM, 49 μCi/μmol). Culture medium (including radiochemical label) was changed daily.

Isolation of lamellar body material

Lamellar body material was isolated after 7 days of culture using methods described previously (15). Two lamellar body fractions were recovered after the final sucrose density gradient centrifugation: *1*) a fraction at the interface between buffer (0.15 M NaCl, 0.1 mM EGTA, 0.01 M diethylmalonic acid, pH 7.4) and buffer + 0.41 M sucrose, and *2*) a fraction at the interface between buffer + 0.41 M sucrose and buffer + 0.65 M sucrose. Only the material recovered at the interface between buffer and buffer + 0.41 M sucrose was used for the analyses reported here. This material has been shown by electron microscopy, analysis of lipid/protein ratio, and analysis of lipid composition to be the more characteristic of lamellar body material (13).

Lipids from the lamellar body material were extracted into chloroform–methanol using modifications (24) of the procedures of Bligh and Dyer (25), and stored at −20°C in chloroform–methanol 2:1 until further use. Quantities of lipid were measured by phosphorus analysis (26).

The yields of lamellar body material we obtained can be summarized as follows. Each 100-mm culture dish of tissue pieces contained the equivalent of one or two fetal lungs (mean 1.4 ± 0.1 lungs (SEM, n = 8)). For each experiment, lamellar body material was isolated from two dishes. The yield of lamellar body material recovered at the interface between buffer and buffer + 0.41 M sucrose ranged from 0.9 to 3.9 μmol lipid P (mean 2.2 ± 0.2 μmol lipid P (SEM, n = 16)) per two dishes of tissue.

The specific radioactivities found in the lamellar body materials were as follows: [1-^{14}C]acetate label (49 μCi/μmol in medium): 0.29 ± 0.04 μCi/μmol lipid P (SEM, n = 8) in lamellar body material; [U-^{14}C]glycerol label (16 μCi/μmol in medium): 0.067 ± 0.006 μCi/μmol lipid P (SEM, n = 4) in lamellar body material; [1-^{14}C]palmitate label (6.4 μCi/μmol in medium): 0.0061 ± 0.0016 μCi/μmol lipid P (SEM, n = 4) in lamellar body material.

Electron microscopy

Tissue pieces of fetal lung were prepared for electron microscopy by fixing in a freshly prepared mixture of 0.7% glutaraldehyde and 0.7% osmium tetroxide in 0.1 M phosphate buffer (pH 7) for 1 hr on ice (27). Tissue pieces were postfixed in 0.25% uranyl acetate in 0.1 M acetate buffer (pH 7) for 30 min on ice.

Lamellar body material was prepared for electron microscopy by centrifugation at 75,000 *g* for 20 min. The pellet was fixed in glutaraldehyde–osmium tetroxide for 1 hr on ice, then fixed in 1.5% tannic acid in 0.1 M phosphate buffer (pH 7) for 30 min at room temperature (28).

Both the tissue pieces and the lamellar body pellets were dehydrated with cold acetone, then with propylene oxide, and then embedded in LX-112 resin (Ladd Research

Industries). Silver-thin sections were obtained with a diamond knife, stained with uranyl acetate and lead citrate, and examined on a Phillips 300 electron microscope.

Preparation of phosphatidylcholine fatty acid methyl esters

Lamellar body phosphatidylcholine (1–2 μmol per experiment) was isolated by preparative TLC on 20 cm × 20 cm silica gel 60 plates, 250 μm thickness, using a solvent system of chloroform–methanol–ammonia 26:14:2. Phosphatidylcholine was extracted from the gel using modifications of the procedures of Bligh and Dyer (24). Fatty acid methyl esters (FAMEs) were prepared by transesterification in boron trifluoride (12% in methanol) (29), extracted into hexane, and stored at −20°C prior to analysis.

Fatty acids at positions *sn*-1 and *sn*-2 of lamellar body phosphatidylcholine were isolated by treatment with phospholipase A_2 as follows. Approximately 1 μmol of phosphatidylcholine was stirred for 2 hr at room temperature in 5 ml of diethyl ether plus 0.5 ml of a solution of 0.1 M sodium borate (pH 7), 20 mM $CaCl_2$, and 0.5 mg of porcine pancreas phospholipase A_2 (30). Lipids were extracted into ethyl acetate–acetone (24) and the reaction products were separated by TLC on 5 cm × 20 cm silica gel 60 plates (250 μm thickness) using a solvent system of chloroform–methanol–ammonia 26:14:2. The fatty acid region (from position *sn*-2) and the lysophosphatidylcholine region (containing fatty acid at position *sn*-1) were scraped from the plate, then treated with boron trifluoride (12% in methanol) (29) to obtain FAMEs.

Gas-liquid chromatography

FAMEs were dissolved in a small volume of carbon disulfide and analyzed by GLC on a 6 ft × ⅛ in column of 10% DEGS-PS (Supelco) at 185°C using a Varian model 3700 gas chromatograph. Chromatographic peaks corresponding to fatty acids 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 + 20:1, and 20:4 were integrated and used for the calculation of phosphatidylcholine fatty acid profiles.

Catalytic hydrogenation

Depending on the amount of FAME recovered after silver nitrate TLC, from 5 to 300 μg of monoenoic fatty acid methyl ester was stirred with 0.5 mg platinum(IV) oxide for 2 hr in 1.0 ml methanol under a positive pressure of H_2 gas (31). The methanol was evaporated and the FAMEs were separated from catalyst by extraction into hexane. FAMEs were dissolved in a small volume of carbon disulfide and analyzed by GLC.

Reverse-phase thin-layer chromatography

RP-18 TLC plates (250 μm thickness, 10 cm × 20 cm) were pre-run in hexane and in acetonitrile, then activated at 70°C for 1 hr. FAMEs in methanol were streaked onto the plate, and the plate was developed twice in acetonitrile at room temperature. Bands of fatty acid methyl ester were visualized by autoradiography (Kodak X-Omat AR film). Two regions of the plate were scraped for recovery of product (see Fig. 4): *1*) a region containing the 14:0 + 16:1 fatty acids, and *2*) a region containing the 16:0 + 18:1 fatty acids. The products were separated from the gel by extracting three times with 2 ml hexane.

Silver nitrate thin-layer chromatography

Silica gel 60 plates (5 cm × 20 cm, 250 μm thickness) were impregnated with silver by dipping in 15% $AgNO_3$ in acetonitrile and activating at 70°C for 1 hr. Samples were applied with hexane and separated by developing twice in toluene at −20°C (32). FAMEs were visualized by autoradiography. Mobilities of the monoenoic FAMEs are reported relative to the mobility of the saturated FAMEs (R_x). For determination of radioactivity, the individual bands were scraped into scintillation vials and counted using Triton–toluene scintillation fluid (33). FAMEs were recovered by extracting the gel scrapings three times with 2 ml diethyl ether and washing the ether extracts twice with 1 M NaCl. The ether extract was dried by passing through a column of anhydrous Na_2SO_4.

The saturated fatty acid fraction isolated by silver nitrate TLC was separated into individual fatty acid classes by HPLC as follows. Methyl esters were hydrolyzed by boiling in 0.6 ml methanol–1 N NaOH 5:1 for 3 hr. The reaction mixture was acidified with HCl and the fatty acids were extracted into diethyl ether. Phenacyl esters were prepared following the procedures of Borch (34) and extracted into hexane. Radiolabeled phenacyl esters were mixed with 10 μg each of unlabeled phenacyl esters of myristate (14:0), palmitate (16:0), and stearate (18:0). These three saturated fatty acid esters were separated by reverse-phase HPLC on an Ultrasphere-ODS (C18) column (Altex) using a 40-ml linear solvent gradient beginning with acetonitrile–water 70:30 and ending with 100% acetonitrile (flow rate 1 ml/min). Elution of phenacyl esters was monitored by UV detection (254 nm). Fractions corresponding to chromatographic peaks 14:0, 16:0, and 18:0 were collected and the radioactivity was determined. Other fractions accounted for less than 1% of the total radioactivity.

Double bond analysis

Methods for determining double bond position are described in a separate publication (23). Briefly, ^{14}C-labeled FAMEs were treated with a mixture of potassium permanganate and sodium periodate, and the resulting monocarboxylic acid and dicarboxylic acid monomethyl ester were converted to phenacyl esters by reaction with 2-bromoacetophenone (phenacyl bromide). The phenacyl ester reaction mixture was injected directly onto the HPLC for analysis. The HPLC was programmed for 10

min of acetonitrile–water 50:50, followed by a 40-min linear gradient to 100% acetonitrile, followed by 10 min of 100% acetonitrile (flow rate 1 ml/min). Fractions of 1.0 ml were collected over most of the chromatogram, with 0.5-ml fractions collected over regions where mono- and dicarboxylic acids were expected to appear. Column fractions were prepared for scintillation counting by adding 3.0 ml CP scintillant (Beckman) per ml of eluant. Regions of the chromatogram containing radioactivity were compared to a standard chromatogram of dicarboxylic acid monomethyl, monophenacyl esters (C6–C11) plus monocarboxylic acid phenacyl esters (C5–C12) obtained on the day of the analysis.

RESULTS

Culture of fetal lung tissue and isolation of lamellar body material

Tissue pieces of fetal rabbit lung were placed into culture at day 23 of gestation. During the culture period, the epithelial cells differentiated to type II cells, which synthesized and secreted lamellar body material. An electron micrograph of a section of tissue after 7 days of culture is shown in **Fig. 1.** The type II cells contained numerous lamellar bodies in the cytoplasm, and considerable lamellar body material was found in the fluid-filled spaces within the tissue pieces. The development and the morphology of the tissue in culture did not differ from that described in detail by other investigators (3, 4).

Lamellar body material was isolated and examined by electron microscopy. Excellent preservation of the multilamellar structure of the material was seen throughout the entire lamellar body pellet. Little evidence of contamination by other subcellular fractions was observed. **Fig. 2** is a micrograph of material located near the center of the pellet.

The phospholipid profile of the lamellar body material from culture has been reported in detail elsewhere (15). To summarize, approximately 80% of the lipid was phosphatidylcholine and 3–5% was phosphatidylglycerol. The disaturated phosphatidylcholine content of the lamellar

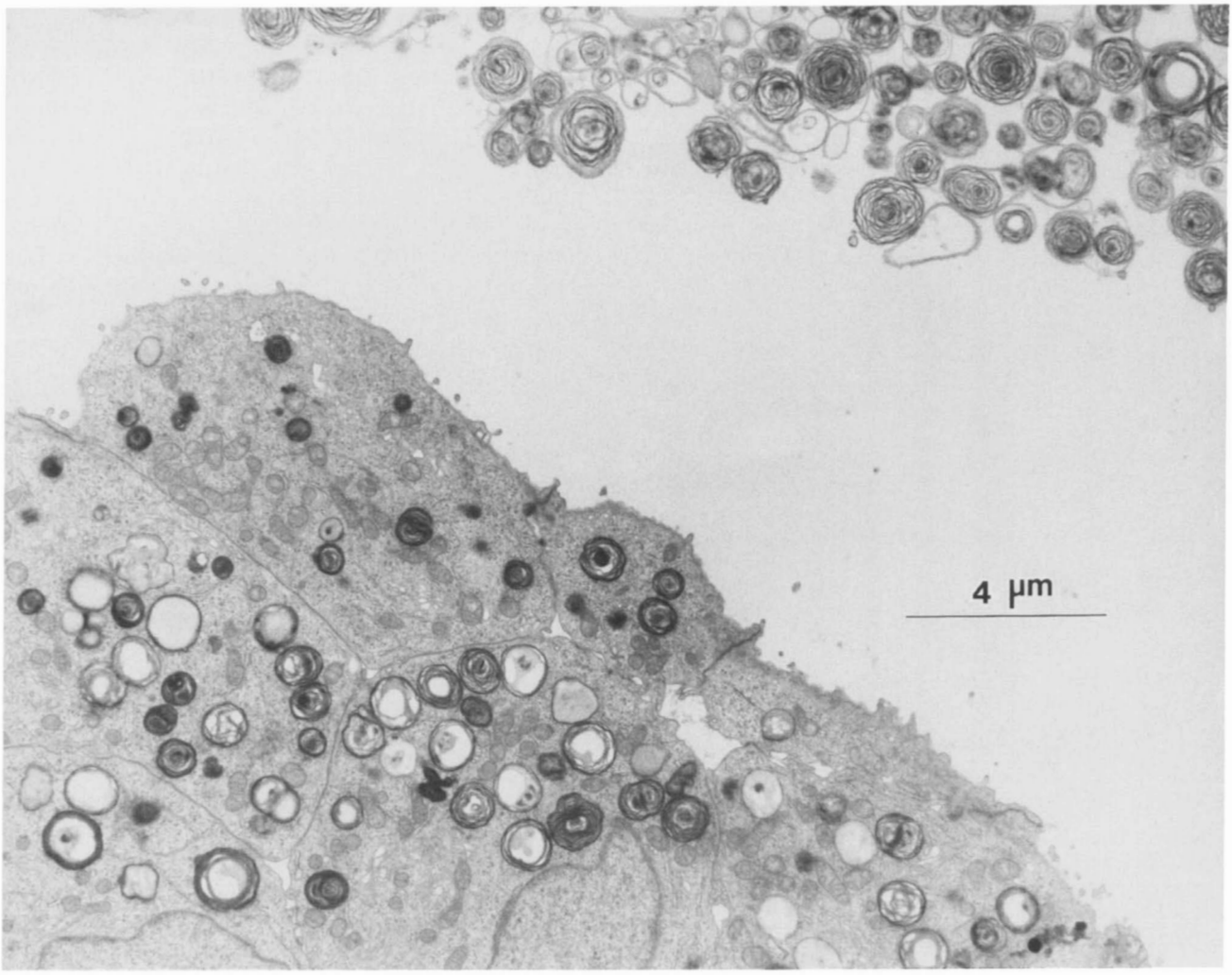


Fig. 1. Electron micrograph of fetal rabbit lung tissue after a 7-day culture period.

body material was approximately 28% of the total lamellar body phospholipid. This value is substantially less than the disaturated phosphatidylcholine content of 48% found in lamellar body material from adult rabbit lung (15).

Isolation of fatty acids from tissue and from lamellar body phosphatidylcholine

Fig. 3 is a schematic diagram of the methods used to isolate and identify the fatty acid methyl esters (FAMEs) from either the tissue homogenate or from the lamellar body phosphatidylcholine. Following lipid extraction and preparative TLC of the lamellar body phosphatidylcholine, the FAMEs were obtained by transesterification using boron trifluoride in methanol. The first chromatographic separation was carried out on reverse-phase TLC plates (RP-18), which separated the FAMEs into three groups as seen in **Fig. 4.** These were *1*) 14:0 + 16:1 FAMEs, *2*) 16:0 + 18:1 FAMEs, and *3*) 18:0 FAME. Two groups, 14:0 + 16:1 and 16:0 + 18:1, were scraped from the plate and extracted into diethyl ether.

Silver nitrate TLC of the above fractions completely resolved the saturated and monoenoic fatty acids from both the lamellar body phosphatidylcholine (**Fig. 5A**) and from the tissue homogenate lipid extract (Fig. 5B). The lamellar body phosphatidylcholine contained three saturated fatty acids: myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0, not scraped from the reverse phase plate). The lamellar body phosphatidylcholine also contained four monoenoic fatty acids: 6-hexadecenoic acid (16:1 *cis*-6), palmitoleic acid (16:1 *cis*-9), oleic acid (18:1 *cis*-9), and *cis*-vacennic acid (18:1 *cis*-11). (Assignment of double bond position is described below.) The fatty acid methyl esters from the tissue homogenate lipid extract contained all of the above saturated and monoenoic FAMEs, plus an additional monoenoic FAME, 8-octadecenoic acid (18:1 *cis*-8) (Fig. 5B).

Confirmation of chain length and determination of double bond position

All FAMEs resolved by silver nitrate TLC (Figs. 5A and 5B) were eluted from silica gel and further characterized. Analysis by GLC indicated that the assignments of

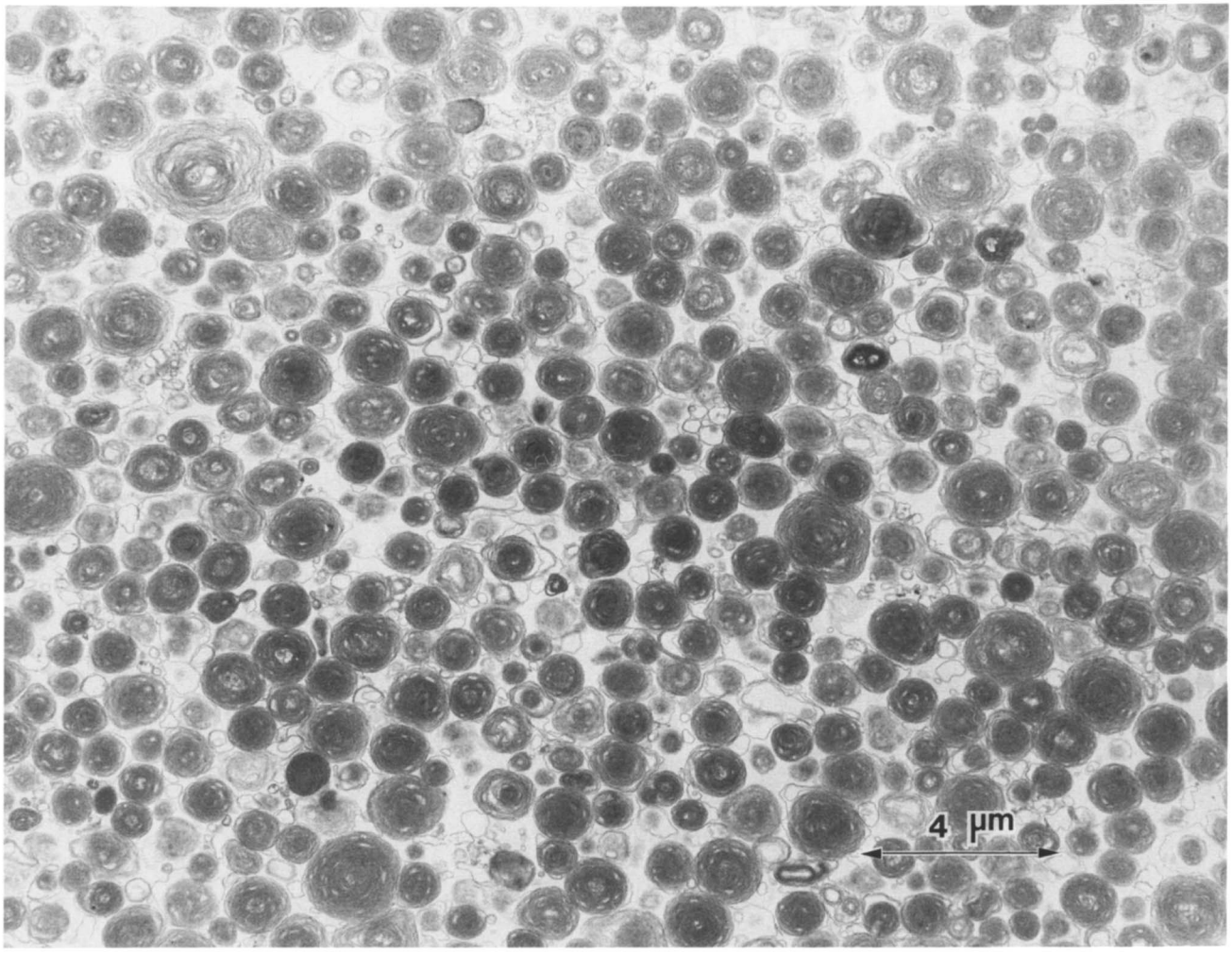


Fig. 2. Electron micrograph of a pellet of isolated lamellar body material from fetal rabbit lung tissue after a 7-day culture period.

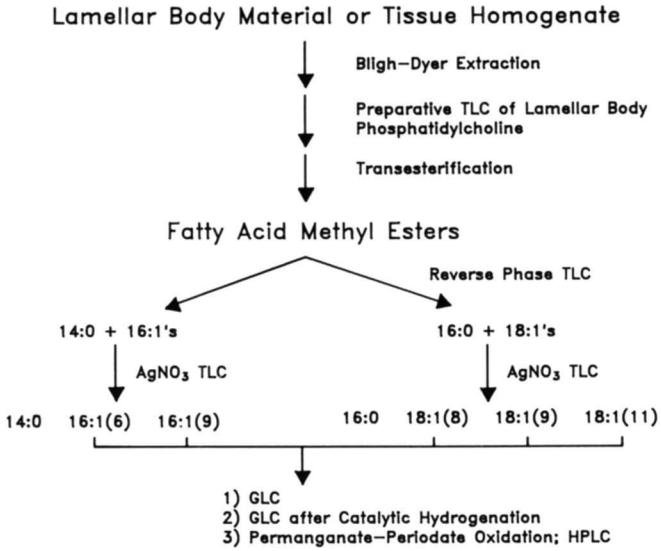


Fig. 3. Outline of methods used to isolate and identify the various saturated and monoenoic fatty acids from lung tissue homogenate and from lamellar body phosphatidylcholine. Cultures were labeled with [1-^{14}C]acetate for 7 days prior to harvest and analysis.

14:0, 16:0, 16:1, and 18:1 were correct. Also, the individual 16:1 and 18:1 monoenoic FAMEs were reduced to the saturated compound by catalytic hydrogenation. By GLC analysis, we confirmed that the two 16:1 FAMEs were reduced to 16:0. All three 18:1 FAMEs were reduced to 18:0.

Double bond position was determined by permanganate–periodate oxidation followed by HPLC of the carboxylic acid phenacyl esters as described in a separate publication (23). **Fig. 6** is an HPLC chromatogram of a mixture of possible phenacyl esters that would be expected from a permanganate–periodate oxidation of monoenoic FAMEs. Dicarboxylic acid monomethyl monophenacyl esters, C6 through C11, and monocarboxylic acid phenacyl esters, C5 through C12, were all resolved on a single chromatogram.

Fig. 7 contains the HPLC chromatograms from the permanganate–periodate oxidations of the ^{14}C-labeled 16:1 and 18:1 FAMEs found in lamellar body phosphatidylcholine and the tissue homogenate lipid extract. The double bond position of palmitoleic acid, 16:1 *cis*-9, was confirmed by the presence of the 9-carbon dicarboxylic acid (monomethyl, monophenacyl ester) and the 7-carbon monocarboxylic acid phenacyl ester (Fig. 7A). Oleic acid was confirmed by the presence of the 9-carbon dicarboxylic acid and the 9-carbon monocarboxylic acid. *cis*-Vacennic acid, 18:1 *cis*-11, was confirmed by the presence of the 11-carbon dicarboxylic acid. The 7-carbon monocarboxylic acid was present in only small amounts, as it was apparently lost during the workup following the oxidation reaction. The reasons for the loss of product are not clear, although we have consistently noted that oxidation of small quantities of FAME (< 100 μg) often results in the preferential loss of the monocarboxylic acid fragment. In any case, determining the chain length of the original fatty acid (18 carbons), and the chain length of the dicarboxylic acid oxidation fragment (11 carbons) was sufficient to assign double bond position. Also, 16:1 *cis*-9, 18:1 *cis*-9, and 18:1 *cis*-11 chromatographed on silver nitrate TLC identically with the commercially available compounds.

The permanganate–periodate oxidation products of the two n–10 FAMEs are shown in Figs. 7D and 7E. The 16:1 fatty acid that on silver nitrate TLC ran below 16:1 *cis*-9 was oxidized to a 6-carbon dicarboxylic acid and a 10-carbon monocarboxylic acid, which assigned the double bond to position 6. The 18:1 fatty acid that chromatographed slightly below 18:1 *cis*-9 was oxidized to an 8-carbon dicarboxylic acid and a 10-carbon monocarboxylic acid, assigning the double bond to position 8. Because this 18:1(8) chromatographed immediately below 18:1 *cis*-9, some oleic acid was apparently scraped from the TLC plate as well, and was found in the analysis of the oxidation products (Fig. 7E).

The position of the 16:1(6) and 18:1(8) fatty acids on silver nitrate TLC establishes the double bond configuration as *cis* and not *trans*. As has been carefully documented by other investigators (35), a *trans* monoenoic fatty acid exhibits considerably more mobility on silver nitrate TLC than the *cis*- isomer. Hence, 16:1 *trans*-6 and 18:1 *trans*-8 would have run considerably ahead of palmitoleic or oleic acids, which was not observed.

Fatty acid profile of lamellar body phosphatidylcholine

When reverse phase TLC (on RP-18 plates) was not performed, the monoenoic FAMEs were separated into four components by silver nitrate TLC: *1*) 18:1 *cis*-11, *2*) 18:1 *cis*-9, *3*) 16:1 *cis*-9 + 18:1 *cis*-8, and *4*) 16:1 *cis*-6. Since 18:1 *cis*-8 was absent from lamellar body phosphatidylcholine (and was a minor component of the tissue homogenate), silver nitrate TLC without prior separation by reverse-phase TLC was sufficient to obtain a profile of

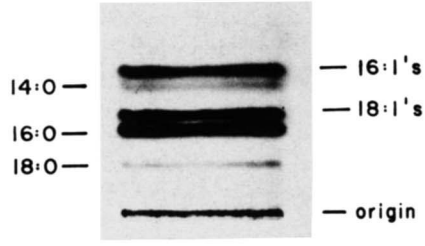


Fig. 4. Reverse-phase thin-layer chromatography of [^{14}C]acetate-labeled fatty acid methyl esters from the lipid extract of the tissue homogenate. ^{14}C-Labeled FAMEs were streaked onto a 10 × 20 RP-18 plate (250 μm thickness), developed twice in acetonitrile, and visualized by autoradiography.

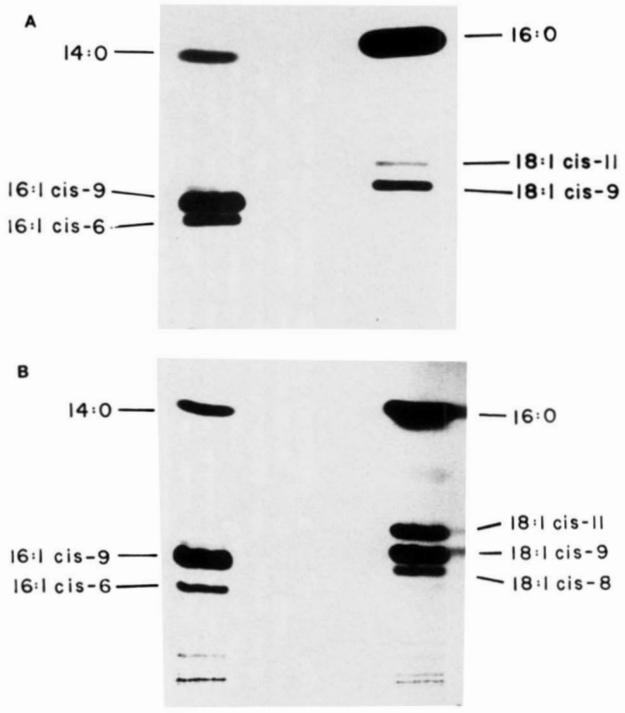


Fig. 5. Silver nitrate TLC of FAMEs from lung tissue homogenate and lamellar body phosphatidylcholine. FAMEs were first separated into fractions of *1*) 14:0 × 16:1's and *2*) 16:0 + 18:1's by reverse phase TLC (Fig. 4). Each fraction was steaked onto a 5 × 20 silica gel 60 TLC plate treated with 15% $AgNO_3$ in acetonitrile. Plates were developed twice in toluene at −20°C and the individual FAMEs were visualized by autoradiography. A: Fatty acid methyl esters from lamellar body phosphatidylcholine. B: Fatty acid methyl esters from the tissue homogenate lipid extract. Mobilities relative to saturated fatty acid (R_x): 16:1 *cis*-6, 0.34; 16:1 *cis*-9, 0.46; 18:1 *cis*-8, 0.41; 18:1 *cis*-9, 0.49; 18:1 *cis*-11, 0.56.

the monoenoic FAMEs. Saturated FAMEs were not resolved by silver nitrate TLC. When necessary, the saturated fatty acids were eluted from the silica gel, converted to phenacyl esters, and analyzed by HPLC.

Fetal rabbit lung tissue in culture was labeled for 7 days with [1-^{14}C]acetate, [U-^{14}C]glycerol, and [1-^{14}C]palmitate. The fatty acid profiles obtained are listed in **Table 1.** Similar profiles were found with all three radiochemical labels. The fatty acid content of the lamellar body phosphatidylcholine was also determined by GLC (Table 1). With the exception of small amounts of 18:2 and 18:3 + 20:1, the profiles obtained by GLC were similar to those obtained by silver nitrate TLC.

Position analysis of the fatty acids of lamellar body phosphatidylcholine

Phosphatidylcholine fatty acid profiles were determined for positions *sn*-1 and *sn*-2 (**Table 2**) in order to understand the reasons for the low content of disaturated phosphatidylcholine. Profiles at each position were determined by *1*) silver nitrate TLC of [1-^{14}C]acetate labeled fatty acid, and *2*) by GLC. For comparison, the fatty acid profiles of freshly isolated lamellar body phosphatidylcholine from adult lung were determined by GLC.

Saturated fatty acids accounted for approximately 90% of the total fatty acid at position *sn*-1 in the lamellar body phosphatidylcholine from both cultured fetal lung and adult lung (Table 2). Palmitic acid was the principal saturated fatty acid. The low disaturated phosphatidylcholine content of fetal lung lamellar body material from culture was explained by the fatty acids found at position *sn*-2. In the fetal lung sample, approximately 30% of the fatty acid at *sn*-2 was saturated. The principal fatty acid at *sn*-2 was 16:1 (55% of total). In adult lung lamellar body phosphatidyl, the principal fatty acid at position *sn*-2 was palmitic acid (54%).

Time course of synthesis and accumulation of fatty acids

Fig. 8 contains the fatty acid profile of fetal rabbit lung tissue with respect to time in culture (0, 2, 4, and 6 days). The striking feature of the profile is the accumulation of 16:1 fatty acids as culture progresses. The level of 16:1 rises from a level of <5% to more than 20% by day 6.

Fig. 9 shows the time course of biosynthesis of the various unsaturated fatty acids, as measured by labeling for 24 hr with [1-^{14}C]acetate and analyzing the FAMEs by silver nitrate TLC. No significant change was observed in the ^{14}C-labeled fatty acid profile throughout the culture period. A high level of palmitoleic acid biosynthesis (ca. 15% of the total fatty acid) was observed from the start of culture. A constant level of synthesis (2–3%) of n–10 fatty acid (16:1 *cis*-6) was observed throughout the culture period.

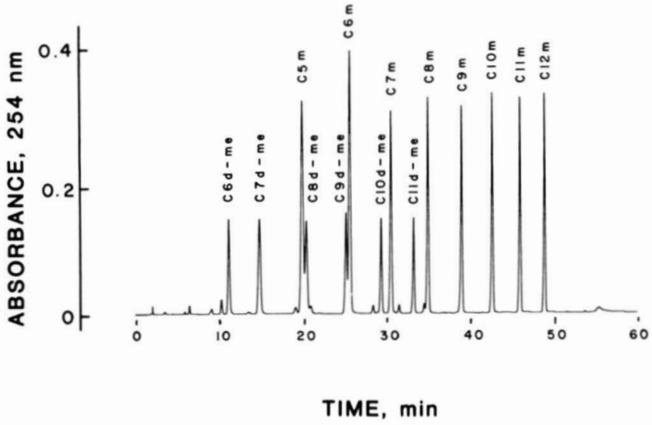
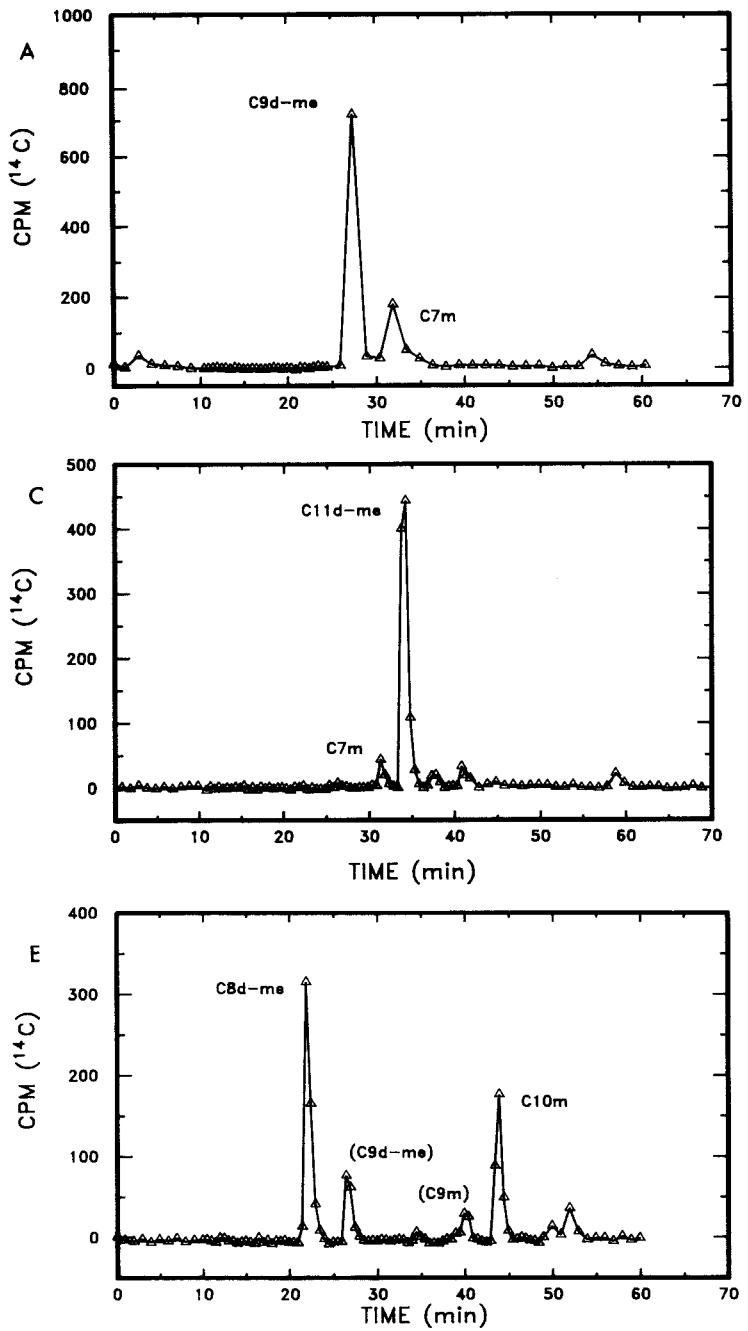


Fig. 6. HPLC chromatogram of a mixture of phenacyl esters of monocarboxylic acids and dicarboxylic acid monomethyl esters. The mixture of phenacyl esters was separated by HPLC using a solvent system of acetonitrile–water 1:1 (10 min), then a linear gradient to 100% acetonitrile (40 min), followed by 100% acetonitrile (10 min). Flow rate, 1 ml/min. Abbreviations indicate the number of carbon atoms in the carboxylic acid chain, and the type of acid: m, monocarboxylic acid; d-me, dicarboxylic acid, monomethyl ester.



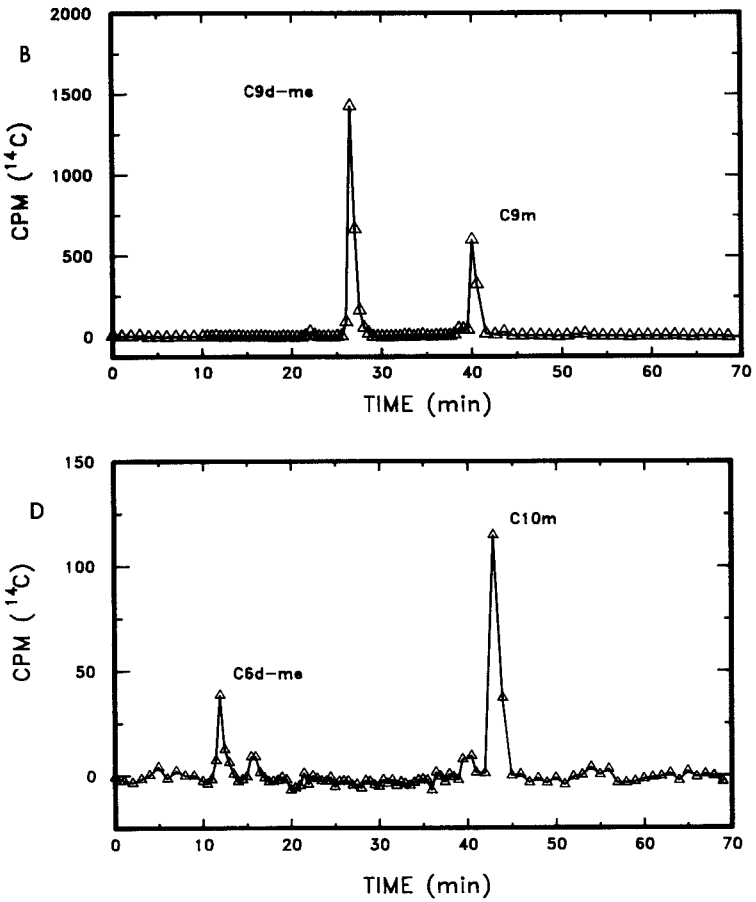


Fig. 7. HPLC chromatograms of the permanganate-periodate oxidation products of the ^{14}C-labeled monoenoic FAMEs from lung tissue in culture. FAMEs were obtained by extraction from silica gel after silver nitrate TLC (Fig. 5). FAMEs were oxidized with permanganate-periodate, and the resulting carboxylic acids were converted to phenacyl esters. HPLC was carried out as described in Methods and in Fig. 6. Either 1.0-ml or 0.5-ml fractions were collected into scintillation vials. Peaks were identified by comparison to a chromatogram of standards (Fig. 6) run the same day of the analysis. A: 16:1 *cis*-9; B: 18:1 *cis*-9; C: 18:1 *cis*-11; D: 16:1 *cis*-6; E: 18:1 *cis*-8.

TABLE 1. Profile of fatty acids of lamellar body phosphatidylcholine from fetal rabbit lung tissue in culture

Fatty Acid	[1-^{14}C]Palmitate	[U-^{14}C]Glycerol	[1-^{14}C]Acetate	GLC
		% of total		
Saturated	58.0 ± 2.2	52.6 ± 1.2	54.8 ± 0.6	58.5 ± 1.4
14:0			1.4 ± 0.7	5.2 ± 0.7
16:0			52.1 ± 0.5	52.4 ± 0.6
18:0			1.3 ± 0.2	0.9 ± 0.1
16:1 *cis*-6	7.0 ± 0.9	3.6 ± 0.1	4.2 ± 0.4	28.3 ± 0.9
16:1 *cis*-9	21.3 ± 2.2	25.6 ± 1.4	25.1 ± 0.9	
18:1 *cis*-9	9.2 ± 1.6	13.1 ± 1.7	11.4 ± 1.1	12.0 ± 0.7
18:1 *cis*-11	4.4 ± 0.2	5.1 ± 0.5	4.6 ± 0.2	
18:2	n.d.	n.d.	n.d.	0.5 ± 0.5
18:3	n.d.	n.d.	n.d.	0.7 ± 0.0

Fetal rabbit lung tissue pieces were placed in serum-free culture for 7 days in the absence of radiochemical label, or in the presence of [1-^{14}C]palmitic acid, [U-^{14}C]glycerol, or [1-^{14}C]acetate. At the end of the culture period, lamellar body phosphatidylcholine was isolated, and the fatty acid methyl esters were prepared. Unlabeled FAMEs were analyzed by GLC. Radiolabeled FAMEs were analyzed by silver nitrate TLC. The saturated fatty acid component obtained by silver nitrate TLC was isolated from the silica gel, converted to phenacyl esters, and analyzed by HPLC. Data are expressed as a percentage of the total FAME detected by GLC or recovered after silver nitrate TLC. Values are mean ± SEM (n = 4); n.d., not detected.

Observation of 16:1 *cis*-6 biosynthesis from the start of culture suggested that this biosynthetic pathway was expressed in the fetal lung tissue in vivo. To test this hypothesis, tissue slices from a day 23 fetal rabbit lung were incubated for 3 hr with 5 μCi [1-^{14}C]acetate in HEPES-buffered minimum essential medium. The 16:1 fatty acids were isolated from the tissue homogenate by reverse-phase TLC followed by silver nitrate TLC as described above. ^{14}C-Labeled 16:1 *cis*-9 and 16:1 *cis*-6 were both detected on the TLC plate.

During these investigations, we tested a variety of compounds and culture conditions in an attempt to reduce the level of 16:1 fatty acids that accumulate in the lamellar body material (Longmuir, K. J., and C. Resele-Tiden, unpublished observations). At present, none of these manipulations has succeeded in reducing the levels of 16:1 fatty acid from those reported here. These additions included hormones and other pharmacologic agents (cortisol, thyroxine, insulin, estrogen, isoproterenol, epinephrine), fatty acid precursors and cofactors (acetate, malonate, biotin), fatty acid–serum albumin complexes (palmitate, linoleate, arachidonate), and sera (fetal calf, newborn calf, adult rabbit). Changes in medium pH were ineffective. Tissue from day 29 gestational age (a time when lamellar body formation takes place in utero) produced the same unusual profile of fatty acids in culture.

TABLE 2. Position of fatty acids on lamellar body phosphatidylcholine from fetal rabbit lung tissue in culture. Comparison with adult lung lamellar body material

	Position *sn*-1			Position *sn*-2		
Fatty Acid	Lung Culture [1-^{14}C]Acetate	Lung Culture GLC	Adult Lung GLC	Lung Culture [1-^{14}C]Acetate	Lung Culture GLC	Adult Lung GLC
			% of total			
Saturated	88.4 ± 1.5	91.3 ± 0.2	92.9 ± 1.9	29.5 ± 2.7	30.7 ± 3.0	56.5 ± 5.5
14:0		7.4 ± 0.8	0.7 ± 0.7		10.5 ± 1.8	2.0 ± 0.4
16:0		83.1 ± 0.6	87.8 ± 2.5		20.2 ± 1.3	54.5 ± 5.8
18:0		0.8 ± 0.1	4.4 ± 0.5		n.d.	n.d.
16:1 *cis*-6	1.2 ± 0.2	6.0 ± 0.2	0.7 ± 0.7	5.4 ± 0.5	54.7 ± 0.6	9.1 ± 2.2
16:1 *cis*-9	6.5 ± 1.0			47.8 ± 2.8		
18:1 *cis*-9	2.1 ± 0.2	2.2 ± 0.3	4.5 ± 1.4	13.2 ± 1.3	14.0 ± 2.4	19.2 ± 1.8
18:1 *cis*-11	1.9 ± 0.3			4.0 ± 0.7		
18:2	n.d.	n.d.	1.4 ± 0.5	n.d.	0.6 ± 0.1	13.9 ± 2.2
18:3 + 20:1	n.d.	0.4 ± 0.1	0.5 ± 0.1	n.d.	0.2 ± 0.1	1.3 ± 0.2

Lamellar body phosphatidylcholine was isolated from fetal rabbit lung in culture and from adult lung in vivo as described in Methods. Phosphatidylcholine was treated with phospholipase A_2. The fatty acid (corresponding to position *sn*-2) and lysophosphatidylcholine (corresponding to position *sn*-1) fractions were separated by TLC, and the fatty acids were converted to methyl esters. Unlabeled FAMEs were analyzed by GLC. Radiolabeled FAMEs were analyzed by silver nitrate TLC. Data are expressed as a percentage of the total FAME detected by GLC or recovered after silver nitrate TLC. Values are mean ± SE (radiolabeling experiments, n = 4; GLC, n = 3); n.d., below the limit of detection.

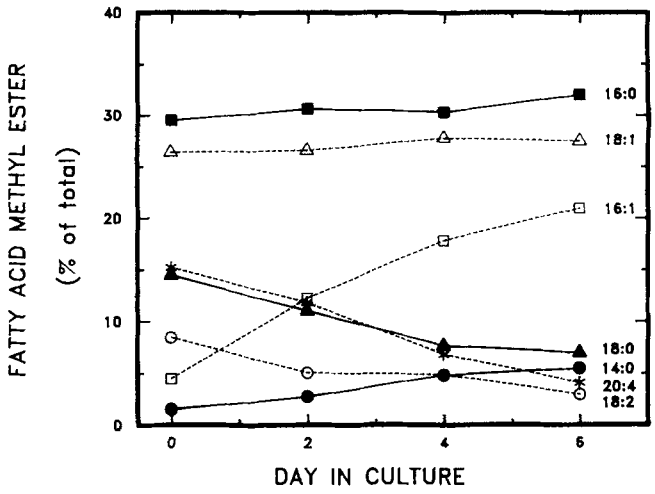


Fig. 8. Time course of accumulation of fatty acids in lung tissue in culture. Fetal rabbit lung tissue, 23 days gestation, was homogenized on days 0, 2, 4, and 6 of culture. FAMEs were prepared from the lipid extract of the tissue homogenate and analyzed by GLC.

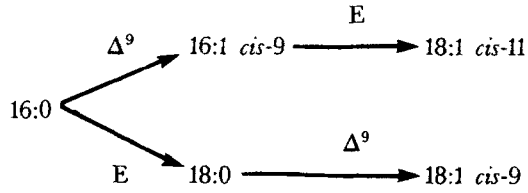
DISCUSSION

Fetal rabbit lung tissue in organ culture produces and secretes a lamellar body material similar in most respects to lamellar body material from fetal and adult lung in vivo. The material has the characteristic multilamellar structure (Fig. 2), and when secreted forms tubular myelin figures (3). It contains 80% phosphatidylcholine (15), phosphatidylglycerol (14, 15), and has a high lipid to protein ratio (13). Biosynthesis of the glycoprotein associated with pulmonary surfactant is expressed in organ culture (16). These similarities to the material in vivo, plus the observations that lamellar body biosynthesis in culture is responsive to hormones and other pharmacologic agents, have made organ culture a valuable system for studying pulmonary surfactant biosynthesis. Surprisingly, the lamellar body material from culture contains less than the expected level of disaturated phosphatidylcholine (15) and an abnormally high content of palmitoleic acid (16:1 *cis*-9). The present investigation utilized sensitive radiochemical labeling methods to identify the various fatty acids produced by fetal lung tissue in culture, and to determine the position of these fatty acids on phosphatidylcholine.

Fatty acid content of lamellar body phosphatidylcholine

Lamellar body phosphatidylcholine from culture contained about 50–60% saturated fatty acid (principally palmitic acid), and the monoenoic fatty acids 16:1 *cis*-6, 16:1 *cis*-9, 18:1 *cis*-9, and 18:1 *cis*-11. In addition, 18:1 *cis*-8 was found in the fatty acids of the tissue homogenate. Fatty acid profiles were virtually the same whether they were measured by *1*) GLC of unlabeled fatty acid, *2*) uptake and incorporation of radiolabeled precursor into fatty acid, or *3*) uptake and modification of exogenous, radiolabeled palmitic acid.

The n-7 (16:1 *cis*-9 and 18:1 *cis*-11) and n-9 (18:1 *cis*-9) fatty acids presumably result from the typical fatty acid elongation and Δ^9 desaturation activities found in most tissues:



E, chain elongation; Δ^9, desaturation at position 9.

Both chain elongation (36) and Δ^9 desaturation (37–39) occur in mammalian lung tissue.

The n-10 fatty acids are a more unusual class of monoenes. In skin, 16:1 *cis*-6 and 18:1 *cis*-8 are present in the secretory product (sebum) of the sebaceous glands (17–20), where they far exceed the levels of palmitoleic and oleic acids. They are the most abundant fatty acids in some plant seed oils (40). They are present in the wax esters of vernix caseosa on the skin of newborn infants (21, 22). The amounts of n-10 fatty acid on skin are correlated with rates of sebum secretion (20, 22), providing evidence that these fatty acids are produced by the skin epithelium rather than acquired from the environment. While it is assumed that 16:1 *cis*-6 is the product of a Δ^6 desaturation enzyme that utilizes palmitoyl-CoA as a substrate (17), this enzyme has not been characterized. (The 18:1 *cis*-8 is then the chain elongation product of 16:1 *cis*-6.) The Δ^6

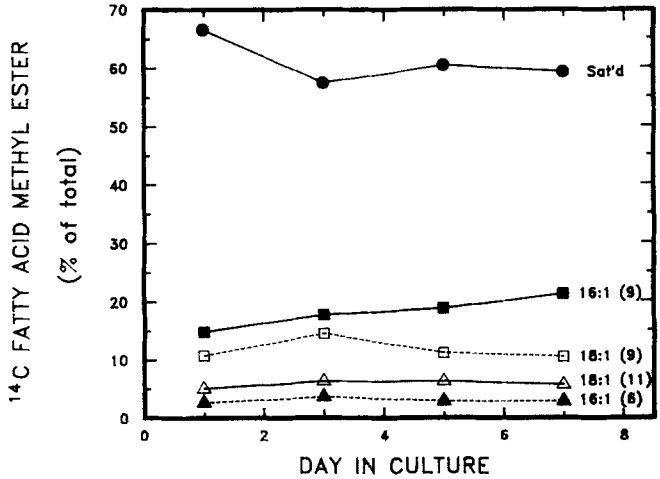


Fig. 9. Time course of biosynthesis of fatty acids in lung tissue in culture. Fetal rabbit lung tissue in culture was labeled for 24 hr with [1-^{14}C]acetate and harvested on the day indicated. FAMEs were prepared from the lipid extract of the tissue homogenate, then separated by silver nitrate TLC into the five classes indicated in the figure.

desaturation enzyme is well characterized in liver tissue, but it utilizes only unsaturated fatty acid for substrate (41).

There are two reports that small quantities of 16:1(6) and 18:1(8) are present in brain tissue in rat (42) and pig (43). There is one report that small quantities of these fatty acids are found in whole rat lung (44). Since those investigations consisted of the analysis of tissue in vivo, it could not be determined whether the fatty acids were the result of de novo fatty acid biosynthesis. Instead, their presence could be the result of incorporation of fatty acid obtained from other tissues or from dietary uptake.

This is the first report of a mammalian tissue, other than skin, that can produce 16:1 *cis*-6 and 18:1 *cis*-8 by de novo biosynthesis. This is also the first report that n-10 fatty acids are incorporated directly into pulmonary surfactant material. The biosynthesis of n-10 fatty acids was observed from the start of culture, and in tissue slices from day 23 fetal lung. Lung organ culture should prove to be a useful system for characterizing this unusual biosynthetic pathway.

The role of n-10 fatty acids in sebum is not established, although it has been noted that substances on the skin surface can retard the growth of microorganisms (17). At this time we cannot assign a role for n-10 fatty acids in pulmonary surfactant. However, it is interesting to note that in mammals these fatty acids are found principally in secreted products that are normally exposed to the environment.

Position analysis of lamellar body fatty acids

Lamellar body phosphatidylcholine from fetal lung in culture correctly contained about 90% saturated fatty acid at position *sn*-1. Position *sn*-2 contained a high level of unsaturated fatty acid (principally palmitoleic acid). The abundance of unsaturated fatty acid at position *sn*-2 reduces the content of disaturated phosphatidylcholine to less than 30% of the total lamellar body phospholipid (15). In vivo, disaturated phosphatidylcholine is 40–50% of the total lamellar body phospholipid in adult rabbit lung (15) and in fetal rabbit lung, day 30 of gestation (45).

Soodsma, Mims, and Harlow (46) have reported that the content of 16:1 fatty acid (in fetal rabbit lung phosphatidylcholine) increases from 5% of the total fatty acid at day 23 of gestation, to 10% at day 30 of gestation. The more dramatic accumulation of 16:1 fatty acid that takes place in culture (from 5% of the total fatty acid at the start of culture to 21% after 7 days) seems to represent an exaggeration of the normal increase in 16:1 fatty acid observed in vivo. To date, a variety of additions to the culture medium, including hormones, fatty acids, and sera, have failed to reduce this apparent overproduction of unsaturated fatty acid.

To produce disaturated phosphatidylcholine, lung tissue can form disaturated diacylglycerol for phosphatidylcholine biosynthesis (47–49). Alternatively, phosphatidylcholine may be modified by a deacylation–reacylation cycle which places a saturated fatty acid at position *sn*-2 (reviewed by Batenburg (50)). Failure to place sufficient saturated fatty acid at position *sn*-2 of phosphatidylcholine could be explained by improper regulation of one or a combination of several steps in fatty acid and glycerolipid biosynthesis. These are *1*) high fatty acid desaturation activity, resulting in excessive cellular levels of unsaturated fatty acid; *2*) inability of the 1-acylglycerophosphate acyltransferase to select saturated fatty acid from a pool containing unsaturated fatty acid; *3*) inability of the cholinephosphotransferase enzyme to select disaturated diacylglycerol from a pool containing unsaturated diacylglycerols; *4*) low phosphatidylcholine deacylation or reacylation activity; and *5*) inability of the lysophosphatidylcholine acyltransferase to select saturated fatty acid from a pool containing unsaturated fatty acid. All of these possibilities can be tested by experiment, and will be the subject of further investigation.

Abnormal fatty acid profiles in lamellar body and surfactant phosphatidylcholine are seen in experiments with animals (51), in human infants with respiratory distress syndrome (52–55), and in the adult respiratory distress syndrome (56, 57). Some of these abnormalities appear to occur because of essential fatty acid deficiencies that result in excessive levels of unsaturated fatty acid, including palmitoleic acid (51, 52). Others report abnormally high levels of 18- and 20-carbon fatty acids in pulmonary surfactant (53–55).

Our studies and those cited above indicate that the control of the lamellar body phosphatidylcholine fatty acid composition is susceptible to the physiologic state of the type II cell. Position *sn*-2 of phosphatidylcholine appears more susceptible than position *sn*-1 to alterations in fatty acid metabolism. Use of various laboratory model systems, such as the culture of lung tissue, should prove valuable for defining the metabolic steps that are most responsible for regulating disaturated phosphatidylcholine biosynthesis.

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