9. Tucker, H. A., Paape, M. J., Sinha, Y. N., Pritchard, D. E., Thatcher, W. W., Proc. Soc. Exptl. Biol. Med. **126**, 100 (1967).

10. Tucker, H. A., Proc. Soc. Exptl. Biol. Med. **116**, 218 (1964).

11. Tucker, H. A., Paape, M. J., and Sinha, Y. N., Am. J. Physiol. **213**, 262 (1967).

12. Talwalker, P. K. and Meites, J., Proc. Soc. Exptl. Biol. Med. **107**, 880 (1961).

13. Dao, T. L. and Gawlak, D., Endocrinology **72**, 884 (1963).

14. Lyons, W. R., Li, C. H., and Johnson, R. E., in "Recent Progress in Hormone Research," Pincus, G. ed., p.219. Academic Press, New York, 1958.

15. Bates, R. W., Milkovic, S., and Garrison, M. M., Endocrinology **74**, 714 (1964).

Received Jan. 10, 1968. P.S.E.B.M., 1968, Vol. 128.

Incorporation of Labeled Palmitate into "Alveolar" and Whole Lung Phospholipids of Fetal and Newborn Lambs* (32949)

TETSURO FUJIWARA AND FORREST H. ADAMS (Introduced by Arthur J. Moss)
Division of Cardiology, Department of Pediatrics, UCLA School of Medcine, Los Angeles, California 90024

The internal surface of the lung is believed to be lined with a surface-active material that stabilizes the alveoli during respiration(1). This material is presumably a lipoprotein complex(1) containing highly saturated phosphatidylcholine(2–4).

The observations that intact mammalian lung rapidly incorporates labeled acetate(5, 6) or palmitate(6–8) into phospholipids suggests a metabolic pattern that may reflect the synthesis in the lung of the phospholipid portion of surface-active material. Autoradiographic studies have shown that labeled acetate and palmitate appear mainly in the large alveolar cells which are thought to be the cellular site of the formation of surface-active material(6). Previous studies by us (9) have shown that dipalmitoyl phosphatidylcholine appears in the fetal alveolar space late in gestation. This is at a time when the phospholipid has increased markedly in concentration in the lung tissue. With the onset of respiration, dipalmitoyl phosphatidylcholine is discharged in large amounts into the alveolar spaces.

The purpose of this study was to determine if labeled palmitate injected into the circulation is incorporated into the phospholipid components of the pulmonary surface-active material of fetal and newborn lambs.

Materials and Methods. Seven fetal and six newborn lambs were studied. All of the fetal lambs were twins, near term, and were delivered by cesarean section under spinal anesthesia. With the placental circulation intact, and the fetus *in utero*, the first of each set of twins (except No. 242A) was given palmitate-1-^{14}C complexed to albumin(10)[1] (100 μC/kg) via coteledyon vein. After 1 hour, the fetus was delivered, the umbilical cord was tied, and the lamb was allowed to breathe normally for 10 min. (One hour was selected because preliminary studies had revealed peak radioactivity of label in lung tissue at 15 min. It was assumed that the label would reach its peak in the "alveolar" phospholipids later.) At the end of 10 min of respiration, the lamb was killed by injection of 1% xylocaine intracisternally.

The second of each set of twins was delivered onto a warm table, taking special precautions to maintain an adequate placental circulation after the mother had first been sedated with intravenous Nembutal and placed on respiratory pump. Labeled palmitate similar to that given its twin was then injected via an isolated jugular vein. After 1 hour, the lamb was killed without allowing it to breathe.

All six of the newborn lambs had delivered spontaneously and ranged in age from 2 hours to 8 days at the time of study. Labeled palmitate was likewise injected into them via

* This work was supported by funds from the United States Public Health Service and the Tuberculosis and Health Association of California.

[1] Palmitic acid-1-^{14}C with a specific activity of 50 mC/mmole was obtained from New England Nuclear Corp., Boston, Massachusetts.

an isolated jugular vein. After 1 hour, the lamb was killed by injection of 1% xylocaine intracisternally.

In all of the animals, immediately after they were killed, the abdominal aorta was severed and the animal was allowed to exsanguinate. The lungs and heart were quickly dissected from the chest in a block and the lungs were separated from the heart and chilled in cold saline. The right major bronchi were tied off and the left lung with the trachea was dissected free. The left lung was sequentially washed with 1200 ml of cold saline through the cannulated trachea. The wash fluid was then centrifuged to remove cells and debris and the supernatant fluid was lyophilized. The reliability of sequential lung washing as a quantitative sampling procedure was reported previously(9).

Analytical procedure. The lipid was extracted from the lyophilizate with $CHCl_3$–MeOH (2:1, v/v) and washed according to Folch *et al.*(11). An aliquot of lipid extract was taken to dryness under nitrogen, dissolved in benzene-acetone (9:1, v/v), and fractionated into neutral lipid, nonacidic, and acidic phospholipids on a DEAE-cellulose acetate column. The lipids were eluted from the column according to Gluck *et al.*(12). Lipid phosphorus was determined on each eluent fraction(13). To estimate the percentage distribution of individual phospholipids, the components were separated by thin-layer chromatography using silica gel H (Merck) in a solvent system of $CHCl_3$–MeOH–H_2O (95:35:4) for nonacidic phospholipids and in $CHCl_3$–MeOH–H_2O (65:25:4) for acidic phospholipids. The spots were made visible by brief exposure to iodine vapor, and were then scraped off the plates. Lipid phosphorus was measured on the scrapings employing 70% perchloric acid digestion(13). Nonacidic phospholipids gave four spots: phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and phosphatidyldimethylethanolamine. After column fractionation, another aliquot of lipids was separated by thin-layer chromatography (TLC) as before. Areas containing phospholipid components were removed from the plates by scraping into scintillation vials. These vials were filled with 15 ml of 4% Cab-O-Sil (Packard) toluene scintillator solution containing 5 gm of 2,5-diphenyloxazole (PPO) and 0.3 gm of 1,4-bis (2-[5-phenyloxazolyl]) benzene (POPOP) per liter(14). The radioactivity of each vial was determined with a Packard Tri-Carb liquid scintillator spectrometer (model 3003) with a counting efficiency of 65.4%.

To determine the location of ^{14}C activity, the phosphatidylcholine was hydrolyzed using snake venom. The phosphatidylcholine was eluted from the scrapings with $CHCl_3$–MeOH (1:1) and evaporated under nitrogen. Crotalus adamanteus lyophilized venom (Ross Allen's Reptile Institute, Inc., Silver Springs, Florida), 2.0 mg in 0.2 ml Tris buffer with 0.002*M* $CaCl_2$ (pH 7.4), was added to 5 ml diethyl ether containing phosphatidylcholine, and was incubated at room temperature for 1 hour. The products were evaporated under nitrogen and extracted with $CHCl_3$–MeOH (1:1). The extracts were separated on TLC as before. The areas containing fatty acid and lysophosphatidylcholine were removed from the plates by scraping into scintillation vials. The radioactivity of each vial was measured as before. To determine the location of ^{14}C activity in the lysophosphatidylcholine, the areas containing this phospholipid were scraped off the plates and eluted with $CHCl_3$–MeOH (1:1, v/v) and then with methanol. After evaporation of the solvent under nitrogen, the lysophosphatidylcholine was hydrolyzed with NaOH in methanol (15). The radioactivity was measured on the fatty acid and water-soluble fractions (glycerophosphorylcholine) as before.

Lipids were extracted from a portion of the lower lobe of the right lung tissue according to Folch *et al.* (11). Fractionation, analytical procedures, and radioactivity measurements were carried out as before. Tissue samples contiguous to those used for lipid extraction were analyzed for dry weight employing a constant temperature at 100°C for 48–72 hours.

Results. Table I summarizes the results of the incorporation of palmitate-1-^{14}C into "alveolar" phospholipids of the 13 fetal and

TABLE I. Incorporation of Palmitic Acid-1-^{14}C into "Alveolar" Phospholipids of Fetal and Newborn Lambs.

			Phosphatidyl choline			Phosphatidyl ethanolamine			Phosphatidyl dimethylethanolamine			Sphingomyelin			Acidic phospholipids[d]		
No.	B.W.[a]	Age	cpm[b]	%[c]	cpm/mg lipid	cpm	%	cpm/mg lipid	cpm	%	cpm/mg lipid	cpm	%	cpm/mg lipid	cpm	%	cpm/mg lipid
242B[e]	4.4	Term-fetus	4810	70	200	550	8	393	800	12	1333	230	3	1150	525	8	952
243B	4.4	Term-fetus	3675	78	157	285	6	259	420	9	840	120	2	1200	218	5	354
244B	5.4	Term-fetus	6640	88	312	200	3	182	400	5	500	110	2	550	200	3	325
245B	3.8	Term-fetus	4200	82	158	200	4	222	460	8	800	100	2	333	236	6	131
243A	4.4	1/6 hour	10,067	88	245	233	4	166	426	4	710	130	1	650	631	6	545
244A	4.3	1/6 hour	21,780	86	369	600	2	277	1640	6	2342	220	1	550	810	3	515
245A	4.4	1/6 hour	12,600	90	253	206	2	108	400	3	667	216	2	432	686	5	437
218	4.0	2 hours	48,424	90	314	940	2	154	1000	2	278	610	1	436	3027	6	628
211	3.4	18 hours	62,650	94	729	622	1	249	1200	2	600	660	1	660	1705	3	485
215	4.1	24 hours	38,687	90	278	501	1	69	1000	2	313	677	1	846	2098	5	466
210	3.4	2 days	79,079	93	316	400	1	26	464	1	63	230	1	135	5229	6	772
216	5.7	3 days	66,420	93	523	660	1	150	1200	2	375	388	1	485	2520	4	591
217	4.3	8 days	60,480	91	400	1000	2	132	1440	2	400	410	1	293	3600	5	720

[a] B.W., body weight.

[b] cpm, counts per minute from left lung.

[c] Percentage of total radioactivity in the "alveolar" phospholipids.

[d] Phosphatidyl serine, phosphatidyl inositol, an unidentified spot, and polyglycerophosphatides are included in this fraction. The radioactivity was expressed as the sum of each component measured.

[e] A and B refer to twins.

TABLE II. Incorporation of Palmitic Acid-1-^{14}C into Whole-Lung Phosphatidylcholine of Fetal and Newborn Lambs.

Animal no.	Age	cpm/mg dry weight[a]	cpm/mg lipid	%[b]
242B	Term-fetus	1704	24,000	86
243B	Term-fetus	2060	27,400	79
244B	Term-fetus	1982	26,113	86
245B	Term-fetus	2130	28,514	88
243A	1/6 hour	2120	27,858	82
244A	1/6 hour	1240	15,696	79
245A	1/6 hour	1060	15,035	82
218	2 hours	2660	34,102	86
211	18 hours	2401	31,592	85
215	24 hours	3630	36,400	84
210	2 days	787	6017	74
216	3 days	586	6846	81
217	8 days	370	4258	78

[a] cpm, counts per minute of right lung.

[b] The percentage of the total radioactivity in whole-lung phospholipids.

newborn lambs. The "alveolar" phosphatidylcholine contained 70–88% of the activity present in the "alveolar" phospholipids from the fetus and 86–94% from the newborn. In the twin fetuses that were allowed to breathe 10 min, the radioactivity of the phosphatidylcholine was two to three times greater than in those that were not allowed to breathe. In the newborn lambs, the activity of the "alveolar" phosphatidylcholine was approximately ten times greater than in the fetus. The specific activity (cpm/mg lipid) of the phosphatidylcholine was slightly higher in the newborns than in the fetuses. The radioactivity of phosphatidyldimethylethanolamine was slightly higher in the fetus than in the newborn lambs.

Table II shows the incorporation of the labeled palmitate into the whole lung tissue phosphatidylcholine of the 13 fetal and newborn lambs. The radioactivity and the specific activity were similar in the fetal and newborn lambs up to 2 days of age, at which time both decreased markedly.

The percentage distribution of the label in both "alveolar" and whole lung phosphatidylcholine molecules is shown in Table III. The label was evenly distributed between the lysophosphatidylcholine and β-fatty acid of both the "alveolar" and whole lung tissue phosphatidylcholine. Since the label was present in only the fatty acids of the lysophosphatidylcholine, one can conclude that the palmitate was evenly incorporated into the α- and β-fatty acids of both "alveolar" and lung tissue phosphatidylcholine.

Discussion. These results demonstrate that fetal and newborn lambs incorporate palmitate into "alveolar" phospholipids, chiefly phosphatidylcholine, a principal component of pulmonary surface-active material. Whole-lung phosphatidylcholine contained 74–88% of the palmitate-^{14}C incorporated into the phospholipids. Furthermore, it can be stated that surface-active material is synthesized in the lung and secreted onto the alveolar spaces of the fetus prior to birth. By 10 min after birth, the radioactivity in the "alveolar" phosphatidylcholine increased threefold, and in 2-day-old newborn lambs, it was approximately ten times greater than in the fetus. These results support our previous findings

TABLE III. Percentage Distribution of Radioactivity in the Lysophosphatidylcholine and Beta Fatty Acids of "Alveolar" and Whole Lung Phosphatidyl Choline Obtained from Fetal and Newborn Lambs (one hour after intravenous administration of palmitate-1-^{14}C).

	"Alveolar"			Whole lung	
	Fetus (4)	10 min (3)[a]	Newborn (4)	Fetus (3)	Newborn (3)
Lysophosphatidylcholine[b]	45 (40–52)	44 (42–45)	49 (49)	47 (40–52)	48 (46–49)
Beta position	55 (48–60)	56 (55–58)	51 (50–51)	53 (48–60)	52 (50–54)

[a] Term lambs breathed for 10 min. The numbers in parentheses indicate the number of animals or range.

[b] Lysophosphatidyl choline was hydrolyzed with NaOH in methanol (15). The radioactivity was present mainly in fatty acid fraction.

that respiration has a profound effect on the liberation or discharge of surface-active material onto the alveolar space (9).

Lung tissue phosphatidylcholine showed high labeling and specific activity up to 24 hours of age but thereafter decreased markedly. Similar results have been obtained from *in vitro* studies of rat lung (16,17). This may reflect a change in the pathways of phospholipid metabolism. The results could be due to a decrease in both the synthesis and degradation of lung tissue phosphatidylcholine after the first day of life. However, some or all of the palmitate-^{14}C incorporated may have been a result of an exchange of only a portion of the molecule. The rate of this exchange might have decreased without altering the total concentration of phosphatidylcholine.

On the other hand, the incorporation of palmitate-^{14}C into the "alveolar" phosphatidylcholine increased with advancing age, and the radioactivity in the "alveolar" phosphatidylcholine constituted only a small fraction of that of the whole lung phosphatidylcholine (Tables I and II). These findings suggest that the phospholipids of surface-active material may be stored in a separate pool with distinct metabolic characteristics. The cellular site of the formation of the surface-active phospholipids has been suggested to be the cytoplasm of the large alveolar cells (6).

The radioactivity was almost evenly distributed between the alpha and beta positions of both the "alveolar" and whole lung tissue phosphatidylcholine (Table III). Elongation of palmitate to other fatty acids of phosphatidylcholine probably occurs in the intact lung (6), but is less active as compared with the liver (18). It is significant in this connection that acetate introduced *in vivo* into adult rabbit was incorporated predominatly into palmitate of lung tissue phosphatidylcholine (6). Similar results have been reported in the lung slices of fetal and newborn lambs (19). Our previous studies (to be published) have shown that the "alveolar" phosphatidylcholine contains more dipalmitoyl phosphatidylcholine than does lung tissue of both fetal and newborn lambs. Therefore, the incorporation of palmitate into both α- and β-fatty acid positions of phosphatidylcholine necessarily would be active. The conversion of palmitate to other fatty acids, if it occurs in the "alveolar" phosphatidylcholine, may be much smaller than that of the lung tissue phosphatidylcholine.

It is of interest that the radioactivity of "alveolar" phosphatidyldimethylethanolamine tends to be higher than that of phosphatidylethanolamine and that the specific activity was also higher than that of phosphatidylcholine in the fetal and newborn lambs that breathed for 10 min after birth. This phospholipid, an intermediate in one pathway of phosphatidylcholine synthesis, has been shown to be a component of the "alveolar" phospholipids of the adult dog (5). Our previous studies have shown that this phospholipid, isolated from lung washings of fetal and newborn lambs, contains more disaturated phospholipids than does the phosphatidylethanolamine (9). The relatively high specific activity present in this phospholipid suggests a methylation pathway in the formation of disaturated phosphatidylcholine of surface-active material.

Summary. The incorporation of labeled palmitate into the phospholipid components of pulmonary surface-active material and whole lung tissue was studied in fetal and newborn lambs. Labeled palmitate was incorporated into the "alveolar" phospholipids. The radioactivity in the "alveolar" phosphatidylcholine was two to three times greater in lambs allowed to breathe for 10 min. In older newborn lambs, the activity was ten times greater than in the fetus.

The radioactivity and specific activity of the whole lung tissue phosphatidylcholine were similar in fetal and newborn lambs up to 2 days of age, at which time both decreased markedly. The radioactivity was evenly distributed between the alpha and beta positions of both the "alveolar" and whole lung tissue phosphatidylcholine.

The radioactivity of the "alveolar" phosphatidylcholine constitutes only a small fraction of the whole-lung phosphatidylcholine. These results indicate that the surface-active material is synthesized in the fetal lung and

secreted onto the alveolar space prior to birth, and is further discharged after the onset of respiration.

The authors thank Professor James F. Mead of the Laboratory of Nuclear Medicine and Radiation Biology (University of California at Los Angeles) for his advice and help, and Mrs. Sara Sipos for technical assistance.

1. Pattle, R.E., Physiol. Rev. **45**, 48 (1965).
2. Brown, E. S., Am. J. Physiol., **207**, 402 (1964).
3. Fujiwara, T., Hirono, H., and Arakawa, T., Tohoku J. Exptl. Med., **85**, 33 (1965).
4. Morgan, T. E., Finley, T. N., and Fialkow, H., Biochim. Biophys. Acta, **106**, 403 (1965).
5. Popják, G., and Beeckmans, N. L., Biochem. J., **47**, 233 (1950).
6. Buckingham, S., Heinemann, H. O., Sommers, S. C., and McNary, W. F., Am. J. Pathol., **48**, 1027 (1966).
7. Havel, R. J., and Felts, J. M., Van Duyne, C. M., J. Lipid Res., **3**, 297 (1962).
8. Naimark, A., J. Appl. Physiol., **21**, 1292 (1966).
9. Fujiwara, T., Adams, F. H., El-Salawy A., and Sipos S., Proc. Soc. Exptl. Biol. Med. (In Press).
10. Fillerup, D. L., Migliore, J. C., and Mead, J. F., J. Biol. Chem. **233**, 98 (1958).
11. Folch, J., Lees, M., and Sloane-Stanley, G. H., J. Biol. Chem., **226**, 497 (1957).
12. Gluck, L., Kulovich, M. V., and Brody, S. J., J. Lipid Res., **7**, 570 (1966).
13. Phillips, B. M., and Robinson, N., Clin. Chim. Acta, **8**, 832 (1963).
14. Snyder, F., and Stephens, N., Anal. Biochem., **4**, 128 (1962).
15. Dawson, R. M. C., Biochem. J., **75**, 45 (1960).
16. Weinhold, P., and Villee, C. A., Biochim. Biophys. Acta, **106**, 540 (1965).
17. Dobiášôva, M., Hahn, P., Drohota, Z., and Dominas, H., Biol. Neonat., **10**, 200 (1966).
18. Elovson, J., Biochim. Biophys. Acta, **106**, 291 (1965).
19. Chida, N. and Adams, F. H., J. Lipid Res. **8**, 335 (1967).

Received Dec. 12, 1967. P.S.E.B.M., 1968, Vol. 128.

Alterations in Hematocrit and Respiratory Rate Induced by Methylene Blue* (32950)

THOMAS P. STOSSEL (Introduced by Robert B. Jennings)
Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611

During an investigation of the effects of methylene blue on the formation of methemoglobin *in vivo* (1), striking and unexpected increases in venous and arterial hematocrit were observed in both dogs and rats. In the present study, hematocrits, respiratory rate, blood pressure, and heart rate were studied in dogs given intravenous infusions of methylene blue. The increases in hematocrit observed previously were confirmed, and striking increases in respiratory rate were observed.

In order to determine whether splenic contraction could account for the observed increase in hematocrit under such conditions, methylene blue was administered to normal and splenectomized rats. Splenectomy abolished the increase in hematocrit indicating that contraction of the spleen is the probable source of the change in erythrocyte concentration. As splenic contraction is believed to result from sympathetic stimulation (2), the effects of drug-induced sympathetic alpha block on the response to methylene blue also were analyzed.

Materials and Methods. Dogs. Three female dogs weighing from 12 to 17 kg were anesthetized with 30 mg of sodium pentobarbital (Nembutal) per kg of body weight. Over a period of exactly 25 min, 50 ml of freshly prepared warm (32–37°C) methylene blue (Merck, USP) solution in isotonic saline was infused by a dual infusion pump (Harvard Apparatus Co., Inc., Dover, Mass.) via a polyethylene catheter inserted into a foreleg vein. In each case the dose was adjusted to 20 mg of

* This work was supported in part by grants from the National Institutes of Health HE 08729, 5-TI-GM-131, and the Chicago Heart Association.