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### Research Article

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Both heterophils and alveolar macrophages incorporated  $^{14}\text{C}$ -labeled choline and palmitate into disaturated lecithins. Liver slices in which only about 1% of the lecithins were disaturated incorporated very little of these precursors into this fraction. Of the palmitate incorporated in vitro into disaturated lecithins by alveolar macrophages, heterophils, and lung slices, 37% was in the 1 position. In disaturated lecithins isolated from pulmonary lavage fluid, alveolar macrophages, and lung of rabbit 8-12 hr after a single intravenous injection of palmitic-1- $^{14}\text{C}$  acid, 45% of the  $^{14}\text{C}$  was in position 1. At earlier times, from 20-240 min after injection, the distribution of  $^{14}\text{C}$  was similar in the samples from lung, but in those from alveolar macrophages and lavage fluid, the percentage in position 1 was slightly lower.

Glycerol- $\text{U-}^{14}\text{C}$  was incorporated into disaturated lecithins by alveolar macrophages and by lung slices in vitro. Both tissues incorporated very little label from ethanolamine or from methyl-labeled methionine into this fraction. All of the data are consistent with the view [...]

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# Synthesis of Dipalmitoyl Lecithin by Alveolar Macrophages

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**ABSTRACT** A reliable, relatively simple method for isolation and quantification of disaturated lecithins is described. In rabbit lung, 34% of the lecithins were disaturated, in alveolar macrophages, 19%. More than 95% of the fatty acids of the disaturated lecithins from lung and alveolar macrophages was palmitic. Hence, the disaturated lecithins from these sources were essentially all dipalmitoyl lecithin.

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Glycerol-U- $^{14}\text{C}$  was incorporated into disaturated lecithins by alveolar macrophages and by lung slices in vitro. Both tissues incorporated very little label from ethanolamine or from methyl-labeled methionine into this fraction. All of the data are consistent with the view that alveolar macrophages synthesize dipalmitoyl lecithin via the cytidine diphosphate–choline pathway.

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## INTRODUCTION

Dipalmitoyl lecithin is believed to be the major surface active component of pulmonary surfactant (1, 2). In order to investigate the pathways of its biosynthesis, it is necessary to separate dipalmitoyl lecithin from the other lecithins present in tissues. We have developed a convenient and reliable procedure for isolating disaturated lecithins, and have studied the incorporation of radio-labeled potential precursors into this fraction by alveolar macrophages, which are the only type of lung cell that can be obtained in relatively homogeneous populations for studies of this type. As reported below, about 20% of the lecithins of alveolar macrophages are disaturated, essentially all dipalmitoyl. In these cells as in lung, the cytidine diphosphate (CDP)<sup>1</sup>-choline pathway appeared to be the major route of synthesis. Some studies were also carried out with heterophils (which constitute less than 10% of the cells in most preparations of alveolar macrophages), and with liver slices in which only about 1% of the lecithins are disaturated.

## METHODS

*Preparation and incubation of tissues.* Tissues were obtained from white New Zealand rabbits (3–4 kg) of both sexes, which were sacrificed by an intravenous injection of sodium pentobarbital, 25 mg/kg, followed by 35 ml of air. Alveolar macrophages obtained by pulmonary lavage with 0.15 M NaCl solution were collected by centrifugation at 150 g at 4°C for 10 min. They were dispersed in the incubation medium (see below) with a siliconized Pasteur pipette, filtered through silk screen, and collected again by centrifugation. Only preparations which contained more than 90% macrophages (differential count of Giemsa stained smear) were used. Confirmation of the purity of the alveolar macrophages and the absence of granular pneumonocytes was obtained by electron microscopic examination of a sample of cells from one of these preparations, using

<sup>1</sup> Abbreviations used in this paper: BHT, 2,6-di-*tert*-butyl-*p*-cresol; CDP, cytidine diphosphate.

techniques previously described (3). None of the 200 cells counted contained desmosomes or the lamellar bodies characteristic of granular pneumonocytes (Type II alveolar epithelial cells).

The heterophils (more than 90% pure) were obtained 16 hr after intraperitoneal injection of 70 ml of autoclaved neutral sodium caseinate solution (150 mg/ml) (Difco Laboratories, Detroit, Mich.) and were washed once with medium before incubation (4). Slices of liver and lung from untreated animals were made using a Stadie-Riggs tissue slicer.

Cells or tissue slices (*circa* 200 mg) were incubated for 1 hr at 37°C in 3 ml of Krebs-Ringer phosphate medium, pH 7.4, containing 10 mM glucose, 2 mg/ml bovine serum albumin (Fraction V from bovine serum, Armour Pharmaceutical Co., Chicago, Ill.), and a radioactive precursor. After addition of 3 volumes of chilled isotonic saline and centrifugation at 500 g for 5 min, the medium was discarded and the sedimented tissue extracted as described below. (After incubation of alveolar macrophages for 1 hr with palmitic-1-<sup>14</sup>C acid, the medium contained less than 1% of the total labeled disaturated lecithin.) Palmitic-1-<sup>14</sup>C acid (55 mCi/mmol), ethanolamine-1,2-<sup>14</sup>C hydrochloride (1.5 mCi/mmol), choline-1,2-<sup>14</sup>C chloride (5 mCi/mmol), and glycerol-U-<sup>14</sup>C (8.4 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Choline-methyl-<sup>14</sup>C chloride (54 mCi/mmol) and L-methionine-methyl-<sup>14</sup>C (53.6 mCi/mmol) were obtained from Amersham-Searle Corp., Arlington Heights, Ill. Palmitic-1-<sup>14</sup>C acid (>96% of radioactivity in palmitate by gas-liquid chromatography) was complexed to defatted albumin (5). The final concentrations of added precursors were 1.9  $\mu$ M palmitate, 10  $\mu$ M choline, 0.9  $\mu$ M methyl-labeled choline, 111  $\mu$ M ethanolamine, 12  $\mu$ M glycerol, and 0.9  $\mu$ M methyl-labeled methionine. Choline-<sup>14</sup>C-labeled lysolecithin was obtained by hydrolyzing murine liver lecithin (96,000 cpm/ $\mu$ mol) with phospholipase A (6). The fatty acids of the labeled lysolecithin contained 0.6% myristic, 37.5% palmitic, 42.0% stearic, 16.2% oleic, and 3.7% linoleic acid.

**In Vivo studies.** Palmitic-1-<sup>14</sup>C acid or palmitic-9,10-<sup>3</sup>H acid (200 mCi/mmol) (New England Nuclear Corp., Boston, Mass.) (95% of tritium in palmitate by gas-liquid chromatography) bound to albumin was injected into the marginal ear vein of six male rabbits. The amount, 10-400  $\mu$ Ci, varied with the time of sacrifice. After sacrifice and pulmonary lavage, pieces of lung were excised, washed with saline, and extracted. The lungs of the animal sacrificed at 20 min were perfused with saline and only the whitened areas analyzed. The pulmonary lavage fluid was centrifuged (250 g for 10 min) at room temperature. The supernatant fluid was lyophilized, taken up in water, and extracted. The cell pellets were pooled and washed twice with 50 volumes of isotonic saline before extraction.

**Lipid analyses.** Samples of cells, tissue, or lavage fluid were extracted overnight at room temperature with chloroform-methanol (2:1, v/v) containing 50  $\mu$ g/ml BHT (2,6-di-*tert*-butyl-*p*-cresol, Ionol, a gift from Shell Oil Company, New York). When phosphorus analyses were not performed, unlabeled lipids from rabbit lung were added to some samples of alveolar macrophages to facilitate visualization of lecithins on thin-layer plates. The phases were separated (7) using 100 mM KCl or, when palmitic-1-<sup>14</sup>C acid was present, 100 mM K<sub>2</sub>CO<sub>3</sub>. The use of K<sub>2</sub>CO<sub>3</sub> did not cause any detectable hydrolysis of disaturated lecithin. The lower phase was washed with 10 mM choline or etha-

nolamine when the incubations contained choline-<sup>14</sup>C or ethanolamine-<sup>14</sup>C.

Lecithins were isolated by chromatography on 250 micron Silica H plates (Analtech, Inc., Wilmington, Delaware) as described by Parker and Peterson (8).<sup>2</sup> In most experiments, plates were exposed briefly to iodine vapor in order to visualize the lecithin fraction. The fatty acid composition and percentage disaturated lecithins of lung lecithins from plates exposed to iodine were not different from those of samples from plates sprayed with 2,7-dichloro-fluorescein. The lecithin areas were scraped into columns packed with glass wool and containing 2.5 ml of chloroform, methanol, water, acetic acid, 50:50:2:1 (v/v). Columns were then washed with 2.5 ml of the same mixture and three 2-ml portions of chloroform, methanol, water, 30:50:5 (v/v). In three experiments 98% of disaturated lecithins and 94% of mixed lecithins from liver were eluted by this procedure.

Saturated lecithins were separated by a modification of previously described methods (2, 10, 11). The eluates were evaporated nearly to dryness under nitrogen at 35°C. 3 ml of theoretical lower phase (7) and 2 ml of the upper phase were added. The solution was mixed, centrifuged, and the lower phase was evaporated to dryness. The lecithins were dissolved in 50  $\mu$ l of CHCl<sub>3</sub> and adducted in 1 ml of a solution of mercuric acetate in methanol for 16 hr in the dark (12). Following addition of 2 ml of chloroform, 2 drops acetic acid, and 0.75 ml water, the solution was mixed and centrifuged. The upper phase containing excess mercuric acetate was discarded. The lower phase was washed three times with theoretical upper phase (7). (All excess mercuric acetate must be removed before chromatography.) Adducted lecithins were separated from the saturated lecithins by chromatography on Silica H plates with chloroform, methanol, 8 M NH<sub>4</sub>OH, 75:25:3.5. Polyenoic, dienolic, and some of the monoenoic lecithins remained at the origin. Most of the monoenoic lecithins were found in a band that migrated more slowly than the saturated lecithins and was clearly separated from them. In eight samples of nonadducted lecithins isolated in this way, 96-100% of the fatty acids were saturated. After mixing with liver lecithins, over 90% of added dipalmitoyl lecithin could be isolated and recovered by this procedure.

In experiments such as that shown in Fig. 1, disaturated lecithin-<sup>3</sup>H (28.4 Ci/mmol) was added to samples at the time of extraction of lipids from tissue, and values for disaturated lecithin were corrected on the basis of its recovery. For this purpose, egg lecithin (Type D-E, Sigma Chemical Co., St. Louis, Mo.) was reacted with tritium gas (New England Nuclear Corp., Boston, Mass.), and the disaturated lecithins purified as described above.

Degradation of isolated disaturated lecithins with phospholipase A (*Crotalus adamanteus* venom, Sigma Chemical Co.) was carried out at least in duplicate (6, 13). Radioactivity in fatty acids, lecithin, and lysolecithin separated by thin-layer chromatography (8) was determined before and after incubation with venom.

**Other materials and methods.** All solvents were reagent grade (J. T. Baker Chemical Co., Phillipsburg, N. J.) and those used to prepare samples for gas-liquid chromatography were redistilled in an all-glass apparatus. The procedure for making the methyl esters of the fatty acids and

<sup>2</sup> In a sample of lecithin isolated by this procedure from alveolar macrophages incubated with palmitic-1-<sup>14</sup>C acid, over 95% of the radioactivity was in lecithin and less than 2% in palmitoyl carnitine (9).

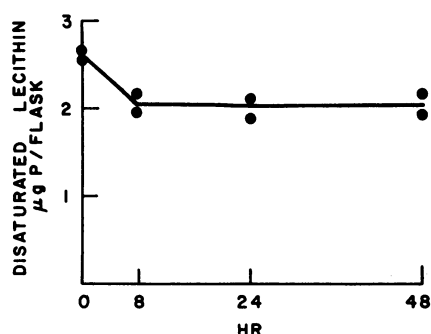


FIGURE 1 Alveolar macrophages in tissue culture. Cells were distributed in 150-cm<sup>2</sup> Petrie dishes and incubated for 2 hr with McCoy's 5A medium, 10% fetal calf serum, penicillin G, 100 U/ml, and streptomycin sulfate, 100 µg/ml. The nonadherent cells were removed by washing with phosphate buffered saline, pH 7.4, fresh medium was added, and the dishes incubated for the indicated time before extraction of cells plus medium. Each point represents the value from one dish corrected for recovery of tritiated disaturated lecithin which was added during the extraction with chloroform-methanol (7).

the conditions for gas-liquid chromatography have been previously described (3). Phosphorus was determined by the method of Bartlett (14). Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (15) with bovine serum albumin as a standard. Supplies used for tissue culture included McCoy's medium 5A (National Institutes of Health Media Unit), fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.), and 150 cm<sup>2</sup> Petrie dishes (Falcon Plastics, Los Angeles, Calif.). Radioactivity of the isolated lecithin fractions was determined by scraping the silica from the thin-layer plates into counting vials and adding dioxane-naphthalene-water scintillation fluid (16). Lecithin (<sup>3</sup>H or <sup>14</sup>C), lysolecithin (<sup>14</sup>C), and palmitic acid (<sup>3</sup>H or <sup>14</sup>C) were completely eluted from silica under these conditions.

## RESULTS

Alveolar macrophages and lung contained considerably more disaturated lecithins per milligrams of protein than did heterophils or liver (Table I). In lung,  $34.2 \pm 0.8\%$

TABLE I  
Phospholipid Content of Tissues

Tissue	Phospholipids	Total lecithins	Disaturated lecithins
<i>µg P/mg protein</i>			
Alveolar macrophages	$12.74 \pm 0.54$	$4.68 \pm 0.19$	$1.06 \pm 0.02$
Lung	$7.88 \pm 0.57$	$3.88 \pm 0.26$	$1.48 \pm 0.11$
Heterophils (WBC)	$3.76 \pm 0.29$	$1.33 \pm 0.08$	$0.22 \pm 0.01$
Liver	$6.21 \pm 0.29$	$2.95 \pm 0.16$	$0.06 \pm 0.01$

The values are the mean  $\pm$ SE for analyses of tissues from three rabbits. These values were calculated from the percentage of the total lipid phosphorus that was lecithin (8) and the percentage of the lecithin that was disaturated for each sample.

(mean  $\pm$ SE,  $n = 10$ ) of the total lecithins were disaturated; in alveolar macrophages  $18.9 \pm 1.4\%$  ( $n = 8$ ), in heterophils  $15.8 \pm 0.5\%$  ( $n = 5$ ), and in liver only  $1.3 \pm 0.2\%$  ( $n = 9$ ). More than 95% of the fatty acids of the disaturated lecithins from alveolar macrophages and lung was palmitic. Hence, the disaturated lecithins from these sources were essentially all dipalmitoyl lecithin.

As shown in Table II, all of the tissues incorporated both palmitic-1-<sup>14</sup>C acid and choline-1,2-<sup>14</sup>C into disaturated lecithins. Incorporation of methyl-labeled choline was comparable to that of the 1,2-labeled compound (data not shown). Alveolar macrophages incorporated more palmitate-<sup>14</sup>C and choline-<sup>14</sup>C per µg lipid phosphorus into all lecithins than did heterophils. For each precursor, however, incorporation into disaturated lecithins as a fraction of incorporation into total lecithins was about the same in the two types of cells. Similarly, in lung slices approximately 40% of the palmitate incorporated into lecithins was in the disaturated fraction and 14% of the choline. In liver slices, on the other hand, disaturated lecithins contained only about

TABLE II  
Incorporation of Precursors into Lecithins In Vitro

Tissue	Palmitic-1- <sup>14</sup> C acid		Choline-1,2- <sup>14</sup> C		Glycerol-U- <sup>14</sup> C	
	Total	Disaturated	Total	Disaturated	Total	Disaturated
<i>cpm/µg lipid P/hr</i>						
Alveolar macrophages	$57.6 \pm 9.9$	$22.8 \pm 3.7$ (4)	$286 \pm 43$	$34.9 \pm 0.6$ (2)	$44.2 \pm 8.4$	$11.5 \pm 2.8$ (3)
Heterophils	$27.6 \pm 2.5$	$14.3 \pm 1.9$ (3)	$52.2$	$7.47$ (1)		
Lung	$16.3 \pm 3.8$	$6.6 \pm 1.5$ (3)	$22.6 \pm 7.8$	$3.23 \pm 1.12$ (2)	$5.48 \pm 0.44$	$1.25 \pm 0.11$ (2)
Liver	$1.19 \pm 0.11$	$0.15 \pm 0.02$ (2)	$1.30 \pm 0.18$	$0.07 \pm 0.01$ (2)		

The number of tissue samples studied (from different animals) is listed in parentheses following the value for disaturated lecithin. Each determination was done at least in duplicate. The values are the mean  $\pm$ SE or one half the range if the number of animals is less than three.

TABLE III  
*Incorporation of 1-Acyl-2-Lysolecithin-<sup>14</sup>C  
into Lecithins In Vitro*

Tissue	Total lecithins	Disaturated lecithins
	<i>cpm/μg lipid P/hr</i>	
Alveolar macrophages	28.07 ± 5.12	1.14 ± 0.13
Heterophils	77.90 ± 9.20	9.53 ± 1.00

Cells were incubated for 1 hr in medium containing 0.5 mM 1-acyl-2-lysolecithin-<sup>14</sup>C and albumin, 30 mg/ml. The values are the means ± SE for samples of alveolar macrophages from four rabbits and heterophils from three.

13% of the palmitate-<sup>14</sup>C and 5% of the choline-<sup>14</sup>C of the total lecithins.

Alveolar macrophages and lung also incorporated glycerol-U-<sup>14</sup>C into lecithins, and about 25% of it was in the disaturated fraction. Lung slices, alveolar macrophages, and heterophils incorporated very little L-methionine-methyl-<sup>14</sup>C (1-hr incubation) or ethanolamine-1,2-<sup>14</sup>C (3-hr incubation) into lecithins. In alveolar macrophages, 95% of the label that was incorporated was in unsaturated lecithins. Liver slices incorporated L-methionine-methyl-<sup>14</sup>C more actively than other tissues, and essentially all the label was in the unsaturated lecithins (data not shown). As shown in Table III and previously reported (17), alveolar macrophages incorporated less than one third as much lysolecithin into lecithins as did heterophils. In the latter cells, about 12% of that incorporated was recovered in the disaturated fraction, in the alveolar macrophages about 5%.

Disaturated lecithins isolated from cells incubated with palmitate-1-<sup>14</sup>C were hydrolyzed with phospholipase A, and the radioactivity in the 1 and 2 positions determined. In samples from macrophages, heterophils, and lung, 35-40% of the <sup>14</sup>C was in position 1 (Table IV). The distribution of radioactivity was not influenced by sex of the donor rabbits or by the time of incubation (15-90 min) (data not shown).

At several times from 20 min to 12 hr after intravenous injection of labeled palmitic acid, the distribution of radioactivity in the extracellular disaturated lecithins of pulmonary lavage fluid was compared with that of alveolar macrophages and of whole lung. As shown in Table V, at early times almost 50% of the label in the lecithins from lung was in the 1 position, whereas in the macrophage and lavage fluid samples 40% or less was in this position. At later times, when the specific activities of the macrophage and lavage fluid disaturated lecithins were higher, the distribution of labeled fatty acids in these samples was not different from that in the lung disaturated lecithins, i.e., in all samples about 45% was in the 1 position.

TABLE IV  
*Distribution of Palmitic-1-<sup>14</sup>C Acid Incorporated  
In Vitro into Disaturated Lecithins*

Tissue	Amount of <sup>14</sup> C in position 1
	%
Alveolar macrophages (7)	38.7 ± 1.9
"Pure" alveolar macrophages* (4)	36.2 ± 0.8
Heterophils (3)	35.7 ± 0.9
Lung (6)	35.7 ± 2.5

The values are the mean ± SE for the number of samples of each tissue (from different animals) shown in parentheses. All determinations were done at least in duplicate. Position 1 is the alpha position in other nomenclature (30).

\* Suspensions consisting of >98% alveolar macrophages were prepared as described by Hurst, Gardner, and Coffin (31).

Several unsuccessful attempts were made to demonstrate accumulation of disaturated lecithins in alveolar macrophages incubated under tissue culture conditions. Different types of medium, different lots and concentrations of fetal calf serum, and different surfaces for growth were used. The initial drop in total disaturated lecithins (Fig. 1) was seen in all experiments including those in which cells only were analyzed, and is consistent with the known ability of these cells to hydrolyze lecithins (18).

## DISCUSSION

All of our data are consistent with the view that alveolar macrophages and lung synthesize dipalmitoyl lecithin via the CDP-choline pathway. In the lung of the adult rabbit we found, as reported earlier by others (19, 20), very little incorporation of label from L-methionine-methyl-<sup>14</sup>C or ethanolamine-1,2-<sup>14</sup>C into any type of lecithin in vitro. Alveolar macrophages also incorporated only small amounts of these precursors into lecithins, and

TABLE V  
*Distribution of Palmitic-1-<sup>14</sup>C Acid Incorporated  
In Vivo into Disaturated Lecithins*

Tissue	Amount <sup>14</sup> C in position 1					
	Sampling time, min after injection					
	20	60	120	240	480	720
	%					
Lung	49	47	44	43	45	44
Lavage fluid	—	32	37	35	44	44
Alveolar macrophage	—	32	40	—	43	48

Each value represents the mean of duplicate analyses of a sample from one rabbit.

95% of that was in unsaturated lecithins. Thus, there was little evidence for synthesis of disaturated lecithins by methylation of phosphatidyl ethanolamine. The demonstrated incorporation of glycerol- $U\text{-}^{14}\text{C}$  and of choline-1,2- $^{14}\text{C}$  into disaturated lecithins by alveolar macrophages and lung would appear to be good evidence for de novo synthesis, but does not exclude the possibility that newly synthesized unsaturated lecithins were rapidly modified to yield disaturated lecithins.<sup>8</sup> The observed distribution of palmitic acid- $^{14}\text{C}$  incorporated into disaturated lecithins in vitro is consistent with this interpretation. In disaturated lecithins isolated from rabbit lung 20-720 min after intravenous administration of the precursor, palmitic-1- $^{14}\text{C}$  acid was almost equally distributed between the 1 and 2 positions. The distribution was similar in the saturated lecithins of alveolar macrophages and lavage fluid after 4 hr. At earlier times when these latter fractions contained smaller amounts of palmitate- $^{14}\text{C}$  (22) there appeared to be a preponderance of label in the 2 position, which could be due to acylation of 1-acyl-2-lysolecithin formed from preexisting lecithins. It has recently been reported that microsomes from dog lung catalyze the acylation of both 1- and 2-lysolecithins (23). The demonstrated incorporation of exogenous 1-acyl-2-lysolecithin into lecithins by alveolar macrophages presumably resulted from acylation since Elsbach (18) found no evidence for synthesis of lecithins by transesterification of lysolecithin in alveolar macrophages.

Because alveolar macrophages can be studied in populations essentially free of other types of cells, it has been possible to designate for the first time at least one specific type of cell from lung that contains dipalmitoyl lecithin in relatively large amounts and can synthesize it. Although the concentration (per milligram protein) of disaturated lecithin in heterophils is only about 20% of that in alveolar macrophages, it is considerably higher than the concentration in many other types of cells (e.g. liver), and the percentage of total lecithin that is disaturated is quite similar in heterophils and macrophages. One wonders whether the disaturated lecithin in these two types of cells is in some way related to their phagocytic function. The role of alveolar macrophages in the production of dipalmitoyl lecithin for pulmonary surfactant remains to be evaluated. Although there are ultrastructural (24) and histochemical (25, 26) similarities between these cells and granular pneumocytes which are believed to be the source of pulmonary surfactant (27, 28), the relationship between the two types of cells is unclear (29). In order to investigate synthesis of dipalmitoyl lecithin by the Type II pneumocytes, it will be necessary to devise methods for separating these cells from other types present in lung.

<sup>8</sup> The extent to which choline might be incorporated by base exchange (21) was not evaluated.

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