De novo fatty acid synthesis and elongation of fatty acids by subcellular fractions of lung

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ABSTRACT
Fatty acid synthesis by subcellular fractions of rabbit lung was studied by measuring the incorporation of either radioactive acetyl coenzyme A or malonyl coenzyme A into long-chain fatty acids. Evidence is presented to support the conclusions that the 95,000 g-supernatant fraction contains the enzymes, i.e., fatty acid synthetase and acetyl coenzyme A carboxylase, necessary for de novo fatty acid synthesis and is capable of synthesizing long-chain fatty acids, probably palmitic acid, under the appropriate conditions. The mitochondrial fraction incorporates the short-chain coenzyme A derivatives into fatty acids predominantly by the elongation pathway. It is suggested that the palmitic acid synthesized in vivo by the de novo fatty acid synthetic pathway, demonstrated in vitro in rabbit lung, may be a source of the lecithin palmitic acid utilized in the synthesis of pulmonary surfactant.

SUPPLEMENTARY KEY WORDS
mitochondria - fatty acid synthetase - palmitate - surfactant

PRESENT INQUIRY into the biochemistry of fatty acid synthesis in the lung is warranted from recent information concerning a material which reduces alveolar surface tension. This surfactant is a lipoprotein composed primarily of phospholipid (1, 2). The major active constituent of pulmonary surfactant is an α-lecithin (3) unique to animal lipids in that it is dipalmitoyl glycerophosphorylcholine (4-6).

Morphological studies suggested that this substance is synthesized by granular pneumocytes (Type II alveolar cell). Electron microscopic studies correlated with surfactant assays have shown a close relationship between levels of pulmonary surfactant and lamellar bodies (osmophilic inclusions) in granular pneumocytes (7-9). These lamellar forms can be seen by electron microscopy as electron-dense cytoplasmic structures which are extruded into the alveoli (10). Some observers believe that there are intermediate forms between mitochondria and lamellar forms, suggesting that mitochondria may be the origin of surfactant (9, 11-13). Others suggest that lamellar forms are nonmitochondrial in origin and may be a storage site of surfactant (7, 14).

There also have been biochemical studies of lung particles which appear to support the hypothesis that mitochondria synthesize surfactant. It was shown that far more acetate-14C is incorporated into fatty acids incubated with a mitochondria-rich fraction than with the 92,000 g-supernatant (15). It was suggested that the mitochondria are the fatty acid source for surfactant. However, it seemed likely that the lung nonmitochondrial system should be quite active and might be the fatty acid source for surfactant since its major product in other tissues, palmitate, is a major constituent of pulmonary surfactant.

The studies in this report show that indeed the nonmitochondrial fatty acid synthesizing system is active in the lung.

MATERIALS AND METHODS
Adult 1.5-2-kg Dutch rabbits of either sex were killed by injection of air into the ear vein. The lungs were removed immediately and chilled in ice-cold 0.01 M Tris–HCl buffer, pH 7.3-7.4, containing 0.25 M sucrose and 0.001 M EDTA (TSE). All separation procedures were performed at 0-4°C. The main arteries and large bronchi were removed from the lungs of two rabbits. The lungs were washed several times in the TSE buffer, and they were then cut into small pieces. The mitocho-
drial fraction was prepared according to the methods of Reiss (16) with the following exceptions: The homogenate was strained through cheesecloth, and the filtrate was centrifuged at 500–600 g for 10 min in an International clinical centrifuge. The supernatant was re- centrifuged at 1450 g for 5 min in a Servall centrifuge.

Mitochondria were removed from the supernatant fraction by centrifugation in a Spinco ultracentrifuge at 13,000 rpm, (11,200 g, rotor no. 40), for 10 min. The creamy white upper portion of the residue, containing free membranes, endoplasmic reticulum, and some mitochondria, was gently removed with a Pasteur pipette. The pellet was resuspended in the sucrose-Tris medium and recentrifuged for 10 min at 15,000 rpm (14,800 g). The upper layer of the precipitate was again discarded. Purity and structural integrity of the mitochondria were repeatedly evaluated by electron microscopy (Fig. 1); the procedures outlined above yielded consistent, satisfactory results. The mitochondria were generally intact, and the preparation was almost free of impurities; however, occasionally short segments of smooth and rough endoplasmic reticulum were present. The remainder of the mitochondrial fraction was re- suspended in a small volume of the sucrose-Tris medium (pH 7.15).

The original supernatant, free of nuclei and the mitochondrial fraction, was centrifuged at 38,000 rpm (95,000 g) in a Spinco ultracentrifuge (rotor no. 40) for 1 hr. The clear 95,000 g-supernatant was made 0.002 M with respect to mercaptoethanol. The microsomes were suspended in the TSE buffer, recentrifuged at 95,000 g for 1 hr, and resuspended in a small volume of the TSE buffer (pH 7.35).

Lungs from two rabbits yielded 9–12 mg of mitochondrial protein and 120–180 mg of 95,000 g-supernatant protein. Roughly equal amounts of mitochondrial and microsomal protein were obtained; individual experiments consistently yielded 16–18 times more supernatant protein than mitochondrial protein per lung. The protein concentration of each fraction was determined...

Fig. 1. Electron micrograph typical of the mitochondrial fractions. Present among the mitochondria were an occasional lamellar body (osmophilic inclusion) of granular pneumocyte origin (arrow) and very few ribosomes (double arrows). Magnification, 45,000 X.
by the method of Lowry, Rosebrough, Farr, and Randall (17). All experiments were carried out on the same day of the separation procedures to minimize loss of enzyme activity. All reactions involving 95,000 g-supernatant protein or microsomal protein contained 0.002 M mercaptoethanol. The rabbit liver fractions were purified by the same procedures as those described for the lung.

The reactions were initiated by the addition of enzyme, i.e., the fractions of tissue obtained by centrifugation, to the reaction mixture and were carried out at 25°C with occasional shaking in 15-ml Kimax culture screw-top tubes. The reactions were stopped after the specified time with 4 volumes of 10% KOH in methanol. Three μmoles of fatty acids (1 μmole of palmitate, 1 μmole of stearate, and 1 μmole of oleate) in a small volume of petroleum ether were then added as carrier. After evaporation of the petroleum ether, the screw-top tubes were tightened and placed in a water bath at 70°C. After 1 hr of occasional vigorous shaking, the tubes were allowed to cool, and the nonsonifiable lipids were extracted twice (v/v) with petroleum ether. The solution was acidified with 5 N HCl to pH 1-2, and the fatty acids were extracted twice (v/v) with n-hexane. Appropriate aliquots were evaporated to dryness in scintillation counting vials, redissolved in 0.1 ml of hexane and 10 ml of Bray’s solution (18), and the radioactivity was counted in a Packard Tri-Carb scintillation counter with an efficiency of 70%. When determining carboxyl carbon radioactivity one-half of each hexane extract was counted in the usual fashion and one-half was subjected to the microdecarboxylation procedure.

**Microdecarboxylation Procedure**

Long-chain fatty acids were decarboxylated by the Schmidt reaction, using the modification of Brady, Bradley, and Trams (19) adapted from the method by Phares (20). The CO₂-Hyamine was counted in 10 ml of toluene containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-[2-(5-phenyloxazole)]-benzene per liter. The counting efficiency and quenching were compared with those when Bray’s solution was used, and the values were standardized with respect to Bray’s solution.

**Radioactive Materials**

Acetyl-1-¹⁴C CoA, malonyl-1,3-¹⁴C CoA, and malonic 2-¹⁴C acid were purchased from New England Nuclear Corp., Boston, Mass. Malonyl CoA and malonyl-2-¹⁴C CoA were synthesized by the procedure of Trans and Brady (21). Acetyl CoA was synthesized according to the method of Simon and Shemin (22). Concentrations of coenzyme A derivatives were measured by hydroxamate assay (23).

ATP, NADPH, NADH, and coenzyme A were purchased from Sigma Chemical Co., St. Louis, Mo. Acetic anhydride, n-hexane, and petroleum ether (bp 38-53°C) were purchased from Fisher Scientific Co., Springfield, N.J. Malonic acid was purchased from Eastman Chemical Products Co., Rochester, N.Y.

**Electron Microscopy**

Pellets of tissue fractions were fixed with cold, buffered 2% gluteraldehyde solution (pH 7.2) for 24 hr, after which the tissue was washed in buffered sucrose solution (24, 25) and postfixed in osmium tetroxide (26). Examination of sections of the Maraglas-embedded (27), lead salt-stained (28) material was carried out in an Elmiskop I electron microscope.

**RESULTS**

**Cofactor Requirements for Lung Fatty Acid Synthesis**

**Pyridine Nucleotide Specificity.** The relative requirements for reduced pyridine nucleotides were similar to those reported for the mitochondrial and soluble fatty acid-synthesizing systems in other tissues (29, 30). As seen in Table 1, the mitochondrial system has a greater dependence upon NADH than NADPH, but NADPH can partially replace NADH. The soluble system has a greater dependence upon NADPH, which can completely replace NADH; NADH can only partially replace NADPH.

| Table 1 Cofactor Requirements for Lung Fatty Acid Synthesis |
|-----------------|-----------------|-----------------|
| System          | Mitochondria CPM | Supernatant CPM |
| Complete 1      | 602             | 755             |
| No NADH         | 322             | 709             |
| No NADPH        | 554             | 418             |
| Complete 2      | 207             | 781             |
| No ATP          | 18              | 679             |
| No malonyl CoA  | 399             | 8               |
| Complete 3      | 1,057           | 270,734         |
| No acetyl CoA   | 1,038           | 94,462          |
| No ATP          | 472             | 281,692         |
| No acetyl CoA, ATP | 347            | 85,298          |

The experiments were carried out as described under Methods in a total volume of 1.0 ml. The complete systems consisted of 1 μmole of NADH, 1 μmole of NADPH, 4 μmoles of ATP, 100 μmoles of potassium phosphate buffer, pH 7.0, and various quantities of CoA substrates and protein. Complete system 1 contained 39 nmoles of acetyl-1-¹⁴C CoA (specific activity, 5,700 cpm per nmole), 80 nmoles of malonyl CoA, and 1.36 mg of mitochondrial protein or 1.46 mg of supernatant protein. Complete system 2 contained 39 nmoles acetyl-1-¹⁴C CoA (specific activity, 6,400 cpm per nmole), 72 nmoles of malonyl CoA, and 1.12 mg of mitochondrial or 1.20 mg of supernatant protein. Complete system 3 contained 39 nmoles of acetyl CoA, 72 nmoles of malonyl-1,3-¹⁴C CoA (specific activity, 13,800 cpm per nmole), and the same amounts of protein as in complete system 2. Results are expressed as cpm of acetyl-1-¹⁴C CoA or malonyl-1,3-¹⁴C CoA incorporated into fatty acids per hour.
**ATP Dependence.** Further similarities to other tissues include the absolute dependence upon ATP for acetyl-1-14C CoA incorporation by the lung mitochondrial system even in the presence of malonyl CoA (Table 1). On the other hand, the soluble system does not need ATP in the presence of both acetyl CoA and malonyl CoA.

The mitochondrial incorporation of acetyl-1-14C CoA into fatty acids is reduced in the presence of malonyl CoA (complete system 2). This is apparently due to dilution of acetyl-1-14C CoA by nonradioactive acetyl CoA formed by decarboxylation of malonyl CoA. In contrast, the supernatant fraction, characteristic of the nonmitochondrial fatty acid-synthesizing system, loses practically all activity without malonyl CoA.

The incorporation of malonyl-1,3-14C CoA into fatty acids by mitochondria is unaffected by the absence of acetyl CoA. The absence of ATP partially depresses mitochondrial fatty acid synthesis from malonyl CoA, and this is not significantly depressed further by the additional absence of acetyl CoA.

The soluble system is greatly stimulated by the addition of acetyl CoA. Incorporation of malonyl-14C CoA is not decreased by the omission of ATP. The prominent malonyl-14C CoA incorporation without exogenous acetyl CoA can be explained by the production of acetyl-1-14C CoA in the presence of malonyl CoA decarboxylase, a cytoplasmic enzyme usually separated with fatty acid synthetase in the first steps of the synthetase purification. It is also important to note the striking quantitative difference in malonyl-14C CoA incorporation into fatty acids between the two systems.

These results establish the presence of a malonyl CoA-dependent fatty acid synthetase in the 95,000 g-supernatant, presumably similar to that described in other tissues and bacteria (30-34).

**Elongation vs. De Novo Fatty Acid Synthesis**

**Supernatant.** Only a small fraction (1-2%) of the total radioactivity incorporated into fatty acids from acetyl-1-14C CoA by the 95,000 g-supernatant was found in the carboxyl carbon. This is indicative of de novo fatty acid synthesis in which acetyl CoA is incorporated only in the first two-carbon fragments of synthesized fatty acids (19, 29, 35). The small amount of radioactivity released during the decarboxylation procedures may represent breakdown of entire fatty acid molecules or actual acetyl CoA carboxylase activity.

It was also necessary to establish the presence of acetyl CoA carboxylase in lung supernatant to prove that de novo fatty acid synthesis may be operative. In the absence of malonyl CoA and without the cofactors for acetyl CoA carboxylase, there is essentially no incorporation of acetyl-1-14C CoA into fatty acids (Table 2). However, in the presence of the known cofactors and an enzyme activator, citrate, there is considerable incorporation into fatty acids. In the presence of malonyl CoA the 95,000 g-lung supernatant protein also incorporated more acetyl-1-14C into long-chain fatty acids after the addition of citrate; the increase was 80-180%.

As further evidence of acetyl CoA carboxylase in lung supernatant, the addition of citrate under both conditions (i.e., with and without malonyl CoA) resulted in a marked increase in radioactive carboxyl carbon. This increase is consistent with the known activation of acetyl CoA carboxylase by citrate in other tissues and would be expected from the in vitro production of malonyl-1-14C CoA; the percentage of carboxyl radioactive carbon would then serve to determine the average length of the fatty acid molecule.

When acetyl-1-14C CoA was incorporated into fatty acids without malonyl CoA, but in the presence of acetyl CoA carboxylase cofactors and citrate, the percentage of carboxyl radioactive carbon in the synthesized fatty acids was 12.8 (Table 3). If palmitate is the product, then 12.5% of the 14C theoretically should be on the terminal carbon.

When malonyl-1,3-14C CoA was the radioactive substrate for supernatant fatty acid synthesis, 13.4% of the incorporated 14C was found on the carboxyl carbon (Table 3). The known de novo pathway for fatty acid synthesis would theoretically yield 14.3% of the 14C in the carboxyl carbon if the product were palmitate. The percentage of carboxyl radioactive carbon was not significantly altered in the presence of citrate. In the absence of exogenous acetyl CoA the results were 13.8% compared with the theoretical value of 12.5%.

In addition, with various concentrations of malonyl CoA and in the presence of 0.01 mM citrate the 14CO2 from the carboxyl was between 10.0 and 14.2% of total 14C.

**TABLE 2  ACETYL COA CARBOXYLASE ACTIVITY**

<table>
<thead>
<tr>
<th>System</th>
<th>% Incorporation of 14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>14.1</td>
</tr>
<tr>
<td>Complete*</td>
<td>19.7</td>
</tr>
<tr>
<td>No KHCO3, MnCl2, citrate</td>
<td>0.003</td>
</tr>
<tr>
<td>No KHCO3, MnCl2, ATP</td>
<td>0.003</td>
</tr>
</tbody>
</table>

All experiments were performed as described under Methods. The complete system contained 1.4 nmoles of acetyl-1-14C CoA (specific activity, 66,000 cpm per mole), 40 μmoles of KHCO3, 3 μmoles of MnCl2, 10 μmoles of ATP, 10 μmoles of Na citrate, 2 μmoles of NADPH, 100 μmoles of potassium phosphate buffer, pH 7.0, and 1.0 mg of 95,000 g-supernatant protein in a total volume of 1.0 ml. Results are expressed as the % of the total acetyl-1-14C CoA incorporated into fatty acids.

* Reaction contained 14 nmoles of acetyl-1-14C CoA and 1.12 mg of protein, but was otherwise similar to complete system described.
incorporated, with an average of 11.9% for five experiments. If the major product of de novo fatty acid synthesis in the lung is palmitate, then the expected percentage of radioactivity in the carboxyl carbon would be 12.5%.

Mitochondria. The mitochondrial fatty acid-synthesizing system was consistently shown to elongate pre-existing fatty acids. When acetyl-1-14C CoA was incorporated into long-chain fatty acids, well over one-half of the radioactivity was on the carboxyl carbon. These results indicate that fatty acid elongation does occur in lung microsomes. Lung microsomes were also similar to microsomes described in other tissues (36, 37) in that they incorporated a greater percentage of malonyl CoA than acetyl CoA into fatty acids.

Optimum Conditions for Fatty Acid Synthesis

pH. Incorporation of acetyl-1-14C CoA into fatty acids by mitochondria was not affected by variation of pH between 6.9 and 7.7. The supernatant was studied under three pH conditions and was found to be most active at pH 6.8, similar to the pH optimum for fatty acid synthesis of other tissues.

Time Course. Supernatant fatty acid synthesis increased in a linear fashion for 1 hr with relatively high concentrations of malonyl-2-14C CoA (24 nmoles per ml) with 1 mg of protein. However, at relatively low concentrations (4.8 nmoles per ml) malonyl CoA became rate-limiting, and maximum levels of incorporation were approached in about 30 min. The mitochondrial system, under the conditions described in Table 4, approached maximum levels of acetyl-1-14C CoA incorporation into fatty acids in 20 min.

Protein Concentrations. Supernatant fatty acid synthesis, as measured by malonyl-2-14C CoA incorporation into fatty acids, increased with increasing the supernatant protein from 0 to 1.0 mg, although the rate of increase diminished above 0.66 mg of protein. The de-
increased rate of incorporation with greater concentrations of protein appears to be due to the rate-limiting effects of available malonyl CoA.

**Comparison of Rabbit Lung and Liver Fatty Acid Synthesis**

**Supernatant.** The incorporation of malonyl-2-14C CoA into fatty acids was compared for lung and liver supernatant protein at different concentrations of malonyl CoA. Fatty acid synthesis by liver was greater than in lung at higher concentrations of malonyl CoA, and liver consistently utilized about 43% of the total malonyl-2-14C CoA in the medium at different malonyl CoA concentrations. It appears that the rate-limiting substance was the “available” malonyl CoA. At low concentrations of malonyl CoA, the lung also incorporated about 43% of the malonyl-2-14C CoA added to the medium. However, at higher concentrations of this substrate, a significantly lower percentage of the malonyl CoA was utilized for fatty acid synthesis in 1 hr.

A valid quantitative comparison of total supernatant (i.e., de novo) fatty acid synthesis cannot be made between the lung and liver systems from these data. However, with the highest concentrations of malonyl CoA studied, the liver synthetase is at least twice as active per mg of protein as the lung system. The average weight of a rabbit’s lungs was 7–8 g, and they contained about 100–110 mg of 95,000 g-supernatant protein. Each rabbit liver contained about 1100 mg of 95,000 g-supernatant protein. Thus, the total fatty acid-synthesizing capacity of the 95,000 g-supernatant fraction appears considerably greater in liver compared with lung.

**Mitochondria.** A few comparisons were made between the liver and lung mitochondrial systems from the same rabbit. The liver contained over 20 times the total mg of mitochondrial protein present in one lung. The liver mitochondria incorporated about four times more acetyl-1-14C CoA into long-chain fatty acids per mg protein than did lung mitochondria.

**DISCUSSION**

In the past ten years, the nonmitochondrial, the mitochondrial, and, more recently, the microsomal fatty acid-synthesizing systems have been intensively studied in many tissues and organisms (38). The particle-free supernatant contains a multienzyme complex which synthesizes long-chain fatty acids, mainly palmitate, by the repeated additions of seven malonyl CoA molecules on an initial acetyl CoA molecule while bound to the synthetase complex. The malonyl CoA is synthesized from acetyl CoA with acetyl CoA carboxylase in what has been shown to be the rate-limiting step for de novo fatty acid synthesis.

The mitochondrial system appears to be involved in at least two different pathways. In the elongation pathway, the acetate of acetyl CoA is added on to preexisting fatty acids which must first be “activated” with ATP. The fatty acids are reduced and generally desaturated, with C-18 and longer unsaturated fatty acids as the major products. A de novo synthesis for fatty acids has also been described involving malonyl CoA, but it appears to be of less significance in mitochondria. Microsomes have recently been shown to synthesize fatty acids by elongation.

Fatty acid synthesis by subcellular fractions in the lung has received little attention. The heterogeneity of cell populations and relative difficulty in homogenizing lung may have added to the general biochemical disinterest in the lung. Nevertheless, the lung is actively involved in lipid metabolism, and the possible role of surfactant in hyaline membrane disease has stimulated interest in surfactant production.

It was already known in 1950 that acetate-1-14C incorporation in the intact lung was predominantly into phospholipids (39); the major phospholipid has since been shown to be lecithin (40). Chida and Adams (41) have shown that in lamb lung slices, acetate-1-14C is incorporated into long-chain fatty acids, primarily palmitic and myristic acids. 87% and 12% of acetate-1-14C incorporation into lecithin fatty acids were in the palmitic acid and myristic acid, respectively.

Tombropoulos, who studied subcellular fractions of rat lung, found that the mitochondria were by far the most active fraction for the incorporation of acetate-1-14C into long-chain fatty acids (15). The supernatant, in striking contrast to other tissues, and microsomes incorporated only a small amount of acetate-1-14C into fatty acids, and the 14C incorporation was considered to be due to cross contamination. He concluded that the mitochondria might be the source of fatty acids for surfactant synthesis.

The present study, however, has shown that the supernatant fraction from rabbit lung homogenates actively synthesizes long-chain fatty acids. Furthermore, the carboxyl carbon assays, along with evidence for high fatty acid synthetase and acetyl CoA carboxylase activities, prove that the lung supernatant does indeed have a de novo fatty acid synthesizing system with palmitate probably as the major product.

This study also demonstrated that rabbit lung mitochondria synthesize fatty acids mainly by the elongation pathway, which appears similar to that described in other tissues. The de novo mitochondrial pathway utilizing malonyl CoA may also be present in the lung, but is of minor quantitative importance.

The mitochondrial incorporation of acetate-1-14C observed by Tombropoulos in rat lung (15) undoubtedly represents elongation of preexisting fatty acids, presum-
ably with C-18 and longer fatty acids being the final products. Palmitate synthesis specifically directed for surfactant in the lung mitochondria of granular pneumocytes by this pathway cannot, obviously, be excluded. It is not entirely clear why the nonmitochondrial pathway studied by Tombropoulos was not observed in rat lung homogenates utilizing acetate-l-14C as the fatty acid precursor. Possible explanations might be deactivation of either the fatty acid synthetase or acetyl CoA carboxylase or inability of the lung to synthesize acetyl CoA from acetate in the supernatant fraction. Nevertheless, it seems likely that rabbit lung synthesizes palmitate for the lecithin moiety of surfactant by the de novo nonmitochondrial pathway, utilizing fatty acid synthetase.

Felts has shown that palmitate-l-14C injected in the blood stream is rapidly incorporated into lecithin in the lung (42). The present study does not prove that the palmitate in surfactant necessarily arises from the existing de novo pathway for fatty acid synthesis in the lung. It appears that in vivo in the lung, lecithin palmitate is derived from the "available" palmitate, whether from the blood stream or the de novo nonmitochondrial pathway, utilizing fatty acid synthetase.

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