Lipid Metabolism by Rat Lung *in vitro*

UTILIZATION OF CITRATE BY NORMAL AND STARVED RATS

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1. The utilization of $[1,5-^{14}\text{C}]$ citrate by lung slices and cell cytosol preparations, and the activities of liver and lung cytosol citrate-cleavage enzyme (EC 4.1.3.8), L-malate–NAD oxidoreductase (malate dehydrogenase, EC 1.1.1.37) and phosphoenolpyruvate carboxylase (EC 4.1.1.32) were examined in normal and starved rats. 2. Lipogenesis from citrate was decreased by approx. 70% in both the phospholipid and neutral lipid fractions of lung slices from starved rats as compared with fed controls. 3. Incorporation of citrate by lung cytosol preparations into fatty acids was decreased by approx. 35% in the starved rats. The apparent inhibition by avidin of fatty acid synthesis was overcome partially by preincubation of lung cytosol preparations with biotin. These results are consistent with the presence in lung tissue of the malonyl-CoA pathway for fatty acid synthesis. 4. Lung citrate-cleavage enzyme activity decreased in rats that had been starved for 72h whereas malate dehydrogenase and phosphoenolpyruvate carboxylase activities remained unchanged. The results suggest that the pattern of utilization of lipid precursors by rat lung may be altered during various nutritional states.

Various studies *in vitro* have demonstrated for mammalian lung the capability of utilizing acetate and glucose for the synthesis of fatty acids (Felts, 1965; Nasr & Heinemann, 1965; Salisbury-Murphy *et al.*, 1966; Chida & Adams, 1967; Scholz & Rhoades, 1971). Active fatty acid synthesis by this tissue is thought to be related in part to the high phospholipid content [primarily (dipalmitoyl) phosphatidylcholine] and rapid turnover of the surface-active material (surfactant) lining the alveolar membranes (Klaus *et al.*, 1961; Tierney *et al.*, 1967). Preformed fatty acids, particularly palmitate, are also utilized by lung for glyceride synthesis (Havel *et al.*, 1962; Felts, 1965; Buckingham *et al.*, 1966; Salisbury-Murphy *et al.*, 1966; Wolfe *et al.*, 1970). These findings, in addition to a report demonstrating the presence of a lipoprotein lipase in mammalian lung (Heinemann, 1961), suggest that this tissue is capable of utilizing various precursors for the synthesis of its lipid components.

The precise role(s) of the lung in obtaining fatty acids for glyceride synthesis during various physiological states is unclear at present. It recently has been reported that phospholipid fatty acid synthesis from glucose was decreased in lung slices of starved rats as compared with the values for fed controls (Scholz & Rhoades, 1971). Normal patterns of glucose utilization by lung slices were observed after re-

feeding starved rats, but they were not accompanied by changes in the activities of enzymes supplying NADPH. Schiller & Bensch (1971) have demonstrated an active pathway for the new synthesis of fatty acids by rabbit lung cytosol preparations. Their studies have shown also the presence of cytoplasmic acetyl-CoA carboxylase activity, suggesting the involvement of the malonyl-CoA pathway in the synthesis of fatty acids in this tissue.

It is well documented that fatty acid synthesis via the malonyl-CoA pathway is affected considerably by the nutritional state of the animal. The importance of citrate in the extramitochondrial synthesis of fatty acids, and its altered metabolism during various nutritional states, has been discussed by Lowenstein (1968). Citrate metabolism by mammalian lung, however, has not been studied. Accordingly, the present studies investigated the utilization of citrate by slices and high-speed supernatant fractions from lung tissue of normal and starved rats. The activities of citrate-cleavage enzyme and additional enzymes indirectly associated with the metabolism of citrate were also investigated. It was considered that the use of dietary manipulations known to alter significantly lipogenesis in other tissues could provide information on possible factors associated with the control and maintenance of the surface-active properties of mammalian lung.

Experimental

Animals and diets

Male Long-Evans hooded rats averaging 300–400 g body wt. were used in all experiments. All rats were housed individually. Temperature, relative humidity and light were controlled at 22°C, 50% and a 12 h light–dark cycle respectively. The animals were fed on a diet of standard laboratory rat pellets (Purina Laboratory Chow; Ralston Purina Co., St. Louis, Mo., U.S.A.) and had free access to water. Starved rats were deprived of food for 72 h.

Preparation of tissue slices and subcellular fractionation procedures

Procedures for killing the rats and perfusing the lungs *in situ* have been described previously (Scholz & Rhoades, 1971). For the lung slice-incubation experiments, tissue slices weighing approx. 100 mg were prepared with a Stadie–Riggs hand microtome. Visceral pleura, major vessels and large bronchi were excluded from the slices. Freshly prepared slices were weighed to the nearest milligram and immediately placed into reaction flasks containing the appropriate substrates and calcium-free Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1957), pH 7.4, maintained at 37°C.

The following procedures were used for preparing lung microsomal and cytosol fractions. Tissue slices of approx. 0.5 mm thickness were prepared as described above and homogenized on ice with a loose-fitting Teflon–glass homogenizer as described by Reiss (1966) in 4 vol. of 0.25 M-sucrose, pH 7.4, containing 1 mM-dithiothreitol and 10 mM-tris–HCl. The initial crude homogenate was then homogenized again on ice in a standard Teflon–glass motor-driven homogenizer. The resulting homogenate was strained through nylon mesh and the filtrate centrifuged at 12000g for 10 min at 2°C. The resulting supernatant was centrifuged at 105000g for 1 h at 2°C. The pellet was washed and resuspended in homogenizing buffer and was considered to be the microsomal fraction; the supernatant was used for assays of lung cytosol enzyme activities. Portions of liver tissue were homogenized in 9 vol. of buffered sucrose and the supernatant resulting from centrifugation at 105000g was used for assay of liver enzyme activities.

Techniques with tissue slices

Lung slices were incubated in replicate for 2 h in 50 ml reaction flasks (Kontes Glass Co., Vineland, N.J., U.S.A.) fitted with a scintillation vial on the side arm. Boiled tissue slices were also incubated and served as blanks. The incubation medium (4 ml) for citrate utilization by lung slices contained 40 μ mol

of potassium citrate, 2 μ Ci of [1,5- 14 C₂]citrate (specific radioactivity 8.9 μ Ci/ μ mol; New England Nuclear Corp., Boston, Mass., U.S.A.), 40 μ mol of glucose and calcium-free Krebs–Ringer bicarbonate buffer, pH 7.4. All flasks were gassed for 5 min with O₂ + CO₂ (95:5) before being shaken in a water bath at 37°C. Further incubation procedures and the isolation and determination of radioactivity in 14 CO₂ and the various lipid fractions have been described (Scholz & Rhoades, 1971).

Cell-free fatty acid synthesis from citrate

The incorporation of radioactivity from [1,5- 14 C₂]citrate into fatty acids by the 105000g supernatant fraction was assayed essentially by the procedure of Spencer & Lowenstein (1962). Incubations were conducted at 37°C for 30 min and were terminated by the addition of 4.0 ml of methanolic 5% (w/v) KOH. Then 2 μ mol of palmitic acid was added as carrier to each tube in 100 μ l of light petroleum (b.p. 30–60°C). After evaporation of the light petroleum the tube contents were saponified for 1 h at 70°C. The tubes were cooled and the non-saponifiable fraction was extracted twice with 5 ml of light petroleum. The tube contents were then acidified with 2.5 ml of 5 M-HCl and the fatty acids extracted twice with 5 ml of light petroleum. The fatty acids were concentrated to dryness and resuspended in a known volume of *n*-hexane. Portions were transferred to counting vials, the *n*-hexane was evaporated and the radioactivity counted in 10 ml of toluene scintillant containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l of toluene. Radioactivity counting was performed in a Nuclear–Chicago scintillation spectrometer and the efficiency was determined by the channels-ratio method.

Assay of enzyme activities

The supernatant fraction remaining after centrifugation at 105000g for 1 h was used for the assay of liver and lung cytosol enzyme activities. Citrate-cleavage enzyme [ATP–citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8] was assayed by the method of Cottam & Srere (1969). Malate dehydrogenase (L-malate–NAD oxidoreductase, EC 1.1.1.37) was assayed by the method of Ochoa (1955) and phosphoenolpyruvate carboxylase [GTP-oxaloacetate carboxylase (trans-phosphorylating), EC 4.1.1.32] was assayed by the method of Chang & Lane (1966) as modified by Ballard & Hanson (1967b).

The protein content in the various fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard.

Results

Citrate utilization by lung slices

The utilization of [1,5-¹⁴C]₂ citrate by rat lung slices as assessed by its oxidation to CO₂ and incorporation into phospholipid and neutral-lipid fatty acids is shown in Table 1. A major portion of the citrate utilized was accounted for by its conversion into CO₂. Lipogenesis from citrate was greater for the phospholipid fatty acid fraction as compared with the neutral-lipid fatty acid fraction and is consistent with the preferential utilization by lung of various lipid precursors for phospholipid synthesis. Lipogenesis from citrate was decreased by approx. 70% ($P = 0.01-0.05$) in both the phospholipid and neutral lipid fractions of lung slices from starved rats as compared with the values for the fed rats.

Fatty acid synthesis from rat lung cytosol preparations

The utilization of citrate for fatty acid synthesis by lung slices (Table 1) and the demonstration of acetyl-CoA carboxylase activity (Schiller & Bensch, 1971) suggests that this tissue is capable of citrate cleavage and lipogenesis in the cell cytosol via the malonyl-CoA pathway. A high-speed supernatant fraction from rat lung was used to examine cofactor requirements for fatty acid synthesis from [1,5-¹⁴C]₂ citrate.

Table 1. *Effect of alterations in diet on citrate utilization by rat lung slices*

The values are given as means $\pm$ S.E.M. of six observations, each of which was determined in replicate. Each value represents nmol of citrate oxidized to ¹⁴CO₂ or its incorporation into phospholipid or neutral-lipid fatty acids/min per g of lung at 37°C. Citrate conversion into glyceride fatty acids was calculated by dividing the radioactivity by one-half of the specific radioactivity of the [1,5-¹⁴C]₂ citrate in the final incubation mixture, for reasons discussed by Spencer & Lowenstein (1962). * $P = 0.01-0.05$ as compared with values for control rats fed *ad lib*.

Dietary status ...	Incorporation of citrate (nmol/g per min)	
	Fed <i>ad lib</i> .	Starved for 72h
CO ₂	20.1 $\pm$ 1.6	16.3 $\pm$ 1.4
Phospholipid fatty acids	0.70 $\pm$ 0.11	0.19 $\pm$ 0.03*
Neutral-lipid fatty acids	0.37 $\pm$ 0.04	0.13 $\pm$ 0.02*

Fatty acid synthesis from citrate in lung cytosol fractions was linear both as functions of time and protein concentration (Fig. 1). Routine incubations were carried out for 30 min and with approx. 1–2 mg of protein. The results presented in Table 2 indicate that omission of NADPH, CoA or ATP, or the addition of avidin to the complete system, decreased fatty acid synthesis from citrate by lung cytosol preparations. The apparent inhibition of fatty acid synthesis by avidin was overcome partially by preincubating lung cytosol preparations with biotin. Fatty acid synthesis as assayed in this system was decreased by approx. 35% in the starved rats as compared with the fed controls. However, the magnitude of the decreased response was not significant ($P = 0.1-0.2$).

Liver and lung enzyme activities

The activities of citrate-cleavage enzyme, malate dehydrogenase and phosphoenolpyruvate carboxylase in liver and lung cytosol preparations of fed and starved rats are presented in Table 3. The

Table 2. *Cofactor requirements of and effect of alterations in diet on fatty acid synthesis from citrate by rat lung cytosol preparations*

When avidin alone was added to the complete system (final amount = 50 μ g) it was preincubated initially for 15 min at 37°C with the enzyme preparation. When avidin and biotin were added to the complete system (final amounts 50 μ g and 0.5 μ mol respectively) they were preincubated together initially for 15 min at 37°C and again for an additional 15 min at 37°C with the enzyme preparation. Individual reactions (30 min) were initiated by the addition of the enzyme preparation, which previously had been preincubated for 15 min at 37°C with no additions, with avidin, or with avidin plus biotin, as required. Additional details are given in the Experimental section and in the legend to Fig. 1. The values for the fed and starved animals are given as means $\pm$ S.E.M. of six observations, for each of which replicate determinations were made.

System	Incorporation of citrate into lung fatty acids (nmol/mg of protein per h)
Complete	2.30
–NADPH	0.38
–CoA	0.61
–ATP	0.31
+Avidin	0.34
+Avidin + biotin	1.39
Complete, fed <i>ad lib</i> .	2.16 $\pm$ 0.40
Complete, starved for 72h	1.41 $\pm$ 0.23

Fig. 1. Conversion of citrate into lung fatty acids as functions of time (▲) and protein concentration (●). The assay system was designed after that of Spencer & Lowenstein (1962) and contained 50 μ mol of glycylglycine buffer, pH 7.5, 25 μ mol of NaHCO_3 , 15 μ mol of ATP, 10 μ mol of potassium citrate (0.1 $\mu\text{Ci}/\mu\text{mol}$), 10 μ mol of MgCl_2 , 5 μ mol of MnCl_2 , 5 μ mol of dithiothreitol, 0.3 μ mol of NADPH, 0.15 μ mol of CoA and enzyme in a total volume of 1.0 ml. Incorporation of citrate into fatty acids as a function of time was determined with 1.9 mg of protein. Incorporation of citrate into fatty acids as a function of protein concentration was determined during a 60 min incubation period. An individual point represents nmol of citrate incorporated into lung fatty acids/time-period or protein concentration specified at 37°C. Additional details are given in the Experimental section. Citrate conversion into fatty acids was calculated by dividing the radioactivity by one-half of the specific radioactivity of $[1,5-^{14}\text{C}]$ -citrate in the final incubation mixture, for reasons discussed by Spencer & Lowenstein (1962).

decreased citrate-cleavage activity ($P = 0.01-0.05$), essentially unchanged malate dehydrogenase activity and increased activity of phosphoenolpyruvate carboxylase ($P = 0.01-0.05$) in liver tissue of starved rats as compared with the fed rats has been observed by others (Kornacker & Lowenstein, 1965; Freedland, 1967; Shrago *et al.*, 1963). The changes in liver citrate-cleavage and phosphoenolpyruvate carboxylase activities parallel the changes in lipogenesis and gluconeogenesis observed in livers of starved rats.

Citrate-cleavage enzyme activity was also detected

in lung cytosol preparations and was observed to decrease ($P = 0.01-0.05$) in rats that had been starved for 72 h (Table 3). This finding is consistent with a decreased conversion of glucose (Scholz & Rhoades, 1971) and citrate (present studies) into glyceride fatty acids in lung tissue of starved rats. Malate dehydrogenase and phosphoenolpyruvate carboxylase activities were not changed appreciably in lung preparations of starved rats from values for fed rats ($P = 0.2-0.5$). In the present studies phosphoenolpyruvate carboxylase activity in liver and lung cytosol preparations was determined by the incorporation of $[^{14}\text{C}]$ bicarbonate into acid-stable products by the method of Chang & Lane (1966) as modified by Ballard & Hanson (1967b). With the liver phosphoenolpyruvate carboxylase determinations, omission of IDP from the incubation mixture essentially removed the capacity of this tissue to fix $[^{14}\text{C}]$ bicarbonate. For the determination of lung phosphoenolpyruvate carboxylase activity, however, omission of IDP from the incubation mixture was without effect on $[^{14}\text{C}]$ bicarbonate fixation. Omission of phosphoenolpyruvate from the reaction mixture gave a radioactivity-incorporation value similar to a zero-time control. The cofactor availabilities and specificities for lung phosphoenolpyruvate carboxylase warrant further investigation.

Discussion

The utilization by mammalian lung of acetate and glucose for glyceride fatty acid synthesis (particularly phospholipid fatty acid synthesis) has been demonstrated (Felts, 1965; Nasr & Heinemann, 1965; Salisbury-Murphy *et al.*, 1966; Chida & Adams, 1967; Scholz & Rhoades, 1971). The subcellular site(s) and precise biochemical pathways involved in lung fatty acid synthesis have not been established conclusively. Evidence has appeared suggesting that lung mitochondria are active in surfactant synthesis in general (Klaus *et al.*, 1962) and in fatty acid synthesis in particular (Tombropoulos, 1964). The report of active fatty acid synthesis by a mitochondria-rich fraction of lung using acetate incorporation into long-chain fatty acids (Tombropoulos, 1964) does not preclude an elongation of existing fatty acyl-CoA derivatives rather than new synthesis. Mitochondrial fatty acid synthesis generally is believed to involve elongation of fatty acyl-CoA derivatives (Wakil, 1961), requires NADH as hydrogen donor (Witpeeters, 1969) and, unlike the microsomal and cytosol fatty acid synthetic systems, appears to be unaffected by starvation (Donaldson *et al.*, 1970).

Schiller & Bensch (1971) have demonstrated acetyl-CoA carboxylase activity in the cell cytosol of rabbit lung preparations and have further reported evidence for the new synthesis of fatty acids via the malonyl-CoA pathway. Their studies have shown also an

Table 3. *Effect of alterations in diet on rat liver and lung cytosol citrate-cleavage, malate dehydrogenase and phosphoenolpyruvate carboxylase activities*

The values are given as means $\pm$ S.E.M. of six observations each. One unit represents enzyme activity catalysing the production of 1 μ mol of measured product/min at 25°C for citrate-cleavage and malate dehydrogenase activities and at 37°C for phosphoenolpyruvate carboxylase activity. **P* = 0.01–0.05 as compared with the corresponding value for control rats fed *ad lib*.

Enzyme	Dietary status	Liver		Lung	
		(units/g)	(units/g of protein)	(units/g)	(units/g of protein)
Citrate-cleavage	Fed <i>ad lib</i> .	0.44 $\pm$ 0.04	6.38 $\pm$ 0.52	0.110 $\pm$ 0.010	3.78 $\pm$ 0.38
	Starved for 72h	0.27 $\pm$ 0.03*	4.01 $\pm$ 0.36*	0.083 $\pm$ 0.006*	2.68 $\pm$ 0.27*
Malate dehydrogenase	Fed <i>ad lib</i> .	159.5 $\pm$ 7.1	2266.8 $\pm$ 96.6	34.1 $\pm$ 1.3	1193.8 $\pm$ 68.4
	Starved for 72h	146.6 $\pm$ 6.9	2052.0 $\pm$ 113.9	33.0 $\pm$ 0.9	1072.8 $\pm$ 48.8
Phosphoenolpyruvate carboxylase	Fed <i>ad lib</i> .	1.97 $\pm$ 0.25	27.3 $\pm$ 3.4	0.071 $\pm$ 0.016	2.46 $\pm$ 0.55
	Starved for 72h	2.78 $\pm$ 0.18*	37.2 $\pm$ 2.3*	0.069 $\pm$ 0.017	2.22 $\pm$ 0.54

elongation mechanism for fatty acid synthesis by rabbit lung mitochondria, suggesting the presence of several lipogenic pathways in this tissue. The utilization of citrate for glyceride fatty acid synthesis in the present studies is consistent with the presence of the malonyl-CoA pathway in the cell cytosol of rat lung. The finding that avidin inhibited citrate incorporation into fatty acids by a high-speed supernatant fraction, and that the apparent inhibition was overcome partially by biotin (Table 2), supports this contention. The lung cytosol preparations in the present experiments were not subjected to prior activation with citrate. Fang & Lowenstein (1967) have demonstrated a marked activation by citrate of lipogenesis in high-speed supernatant fractions of rat liver. This effect of citrate is associated with acetyl-CoA carboxylase activation (see Lowenstein, 1968). It appears possible that greater rates of fatty acid synthesis from citrate would have been observed had lung cytosol preparations been incubated with citrate before initiation of the assays. In the present studies, however, it was desired to demonstrate fatty acid synthesis from citrate by rat lung cytosol preparations, rather than to examine optimum incubation conditions *in vitro*. The linear rate of citrate incorporation into fatty acids as a function of incubation time (Fig. 1) agrees favourably with the findings of Fang & Lowenstein (1967), who used a similar incubation system without prior activation with rat liver cytosol preparations.

It was reported previously that glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-isocitrate dehydrogenase activities of lung cytosol were not affected by starvation and re-feeding (Scholz & Rhoades, 1971). 'Malic' enzyme activity was not detected in lung cytosol preparations, suggesting that the interconversion of NADH and NADPH via NAD- and NADP-linked malate dehydrogenases was not operative in this tissue. Further attempts to detect 'malic' enzyme

activity in subcellular locations of lung other than the cell cytosol were undertaken in the present studies but none was found (results not shown). These results suggest that sufficient NADPH-generating capacity is present in rat lung to support fatty acid synthesis in the cell cytosol and that the activities of lung NADPH-generating enzymes do not change during dietary conditions known to alter markedly the activities of these enzymes in other tissues. The presence of citrate-cleavage enzyme activity (Table 3) and the apparent absence of 'malic' enzyme activity in lung cytosol raises the question of the fate of the cytoplasmic oxaloacetate formed.

Ballard & Hanson (1967*a,b*) have reported the absence of 'malic' enzyme activity and the presence of citrate-cleavage enzyme activity in foetal rat liver. These investigators found that the activity of citrate-cleavage enzyme parallels changes in hepatic lipogenesis and suggested that the oxaloacetate formed could enter the mitochondria to support citrate synthesis. Patel *et al.* (1971) have considered in detail the metabolic fate of oxaloacetate formed by citrate cleavage in rat adipose tissue. Although the enzymes associated with oxaloacetate utilization in rat lung cytosol have not been studied in detail, the results presented in Table 3 suggest two possible metabolic routes: (1) the conversion of oxaloacetate into malate via malate dehydrogenase; malate possibly could re-enter the mitochondria to support citrate synthesis or act in the citrate-transport system as described by Chappell *et al.* (1967); (2) the conversion of oxaloacetate into phosphoenolpyruvate via phosphoenolpyruvate carboxylase. The physiological significance of the apparent phosphoenolpyruvate carboxylase activity in rat lung cytosol preparations is unclear at present; however, it is unlikely that rat lung participates in gluconeogenesis, since significant glucose 6-phosphatase and fructose 1,6-diphosphatase activities have not been detected

in this tissue (R. W. Scholz, unpublished work). The possibility that rat lung phosphoenolpyruvate carboxylase functions in glyceroneogenesis, as has been reported for adipose tissue (Ballard *et al.*, 1967; Reshef *et al.*, 1969, 1970), remains to be investigated.

It is emphasized that the quantitative importance of fatty acid synthesis by lung has not been established. In addition to utilizing various precursors for fatty acid synthesis, mammalian lung also actively incorporates palmitate *in vivo* into phospholipid and neutral lipid fractions of lung lipids (Felts, 1965; Buckingham *et al.*, 1966; Harlan *et al.*, 1966). The preferential utilization by lung of various precursors for phospholipid synthesis (as compared with neutral-lipid synthesis) is consistent with the high phospholipid content and turnover of the surface-active layer that lines the alveolar membranes (Klaus *et al.*, 1962; Tierney *et al.*, 1967). The possibility also exists that rat lung relies more heavily upon the uptake from the pulmonary circulation of preformed fatty acids (as opposed to new fatty acid synthesis) for glyceride synthesis during starvation. In this context we have observed increased utilization of glycerol by rat lung *in vivo* during starvation as compared with the fed state (Scholz *et al.*, 1971). The capability of rat lung to alter the pattern of utilization of various precursors for phospholipid synthesis would constitute a metabolic adaptation of value for the maintenance of the surfactant system.

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