

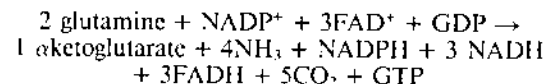
Malic Enzyme Activity in Adult and Newborn Rat Lung

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ABSTRACT. Using a sensitive technique measuring $^{14}\text{CO}_2$ production from radiolabeled malate, we examined malic enzyme activity in both adult and newborn rat lung tissue and in L2 cells, a cell culture line of type II pneumocytes. Malic enzyme was present in both cytosolic and mitochondrial fractions. Time course experiments demonstrated a linear rate after the initial 10 min, up to 30 min. The optimal pH in the cytosolic fraction was 8.0, whereas maximal mitochondrial malic enzyme activity occurred at pH 7.0. The mitochondrial fraction exhibited biphasic kinetics over the 200-fold range of concentrations examined. The high-affinity K_m was 0.16 mmol with V_{max} of 7.11 nmol/mg protein/min. The low-affinity K_m was 6.95 mmol, with V_{max} of 31.82 nmol/mg protein/min. In the cytosol there was a single K_m of 0.30 mmol and V_{max} of 5.95 nmol/mg protein/min. In paired experiments examining differences between 1-d-old and adult rat lung, significantly higher total and mitochondrial malic enzyme activity occurred in the newborn as compared with the adult. Malic enzyme activity was also present in the L2 cells. The finding of malic enzyme activity in the lung suggests that cytosolic malic enzyme may play a role in generating NADPH needed in the lung for fatty acid synthesis. These findings of developmental differences in malic enzyme activity suggest that alternate substrates such as anaplerotic amino acids may be used in the young animal as energy substrates by way of the tricarboxylic acid cycle. (*Pediatr Res* 35: 589-593, 1994)

been reported by several groups (3-5) to play a pivotal role in the four-to-three carbon shunt or "malate oxidation shunt" (Fig. 1). For glutamine to be completely oxidized, some of the accumulating malate must be converted to pyruvate, and this step would require malic enzyme. Without the presence of malic enzyme, the oxidation of glutamine would not be complete because, in the subsequent step, two carbons are added from acetyl CoA, and two carbons are liberated in the form of CO_2 , with no net oxidation of the four carbons of malate. Through the malate oxidation shunt, malic enzyme produces a three-carbon moiety (pyruvate) that may be returned to the tricarboxylic acid cycle after its conversion to acetyl CoA in the mitochondria (Fig. 1). This step allows for the complete oxidation of any anaplerotic compound as an alternate energy source for glucose. The balanced stoichiometry is:



where FAD is flavin adenine dinucleotide, GDP is guanosine diphosphate, FADH is the reduced form of flavin adenine dinucleotide, and GTP is guanosine triphosphate. For example, several studies have shown that glutamine can be used as an alternate energy substrate in both developing and mature brain tissue and other organs (4-7).

Recent results from our laboratory suggest that glutamine may be a key energy substrate in newborn rat lung (8). Therefore, we initiated studies to characterize malic enzyme in lung tissue because little is known about the subcellular localization and regulation of the enzyme in developing lung. Using a sensitive assay developed in our laboratory, we characterized malic enzyme activity in the lung including enzymatic kinetics and subcellular localization, as well as the effect of different malate concentrations and pH. We also compared malic enzyme activity in newborn and adult rats. In addition, we examined enzyme activity in a cell culture line of type II pneumocytes.

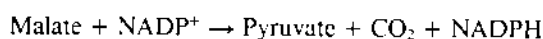
MATERIALS AND METHODS

Materials. Biochemicals and reagents—malic acid, NADP, sucrose, Tris-HCl, and potassium EDTA were purchased from Sigma Chemical Company (St. Louis, MO). The fluor used for liquid scintillation counting (Ready Solve EP) was purchased from Beckman Instruments, Inc. (Fullerton, CA). Solutions for the Pierce BCA microreagent protein assay were purchased from Pierce (Rockford, IL). All reagents and chemicals were of highest analytical grade, and reagent grade water was used to prepare all reagent solutions. The radioactive compound L-[U- ^{14}C] malic acid (53 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). Culture dishes (Nunc) were purchased from Vanguard International (Neptune, NJ). Culture media (minimum essential medium with Earle's salts and nonessential amino acids), antibiotic and antimycotic solution and fetal

The lung is the site of extensive fatty acid synthesis that is required for both the production of the phospholipids used in surfactant and neutral lipids used in the structural membranes of lung tissues (1). The high rate of fatty acid synthesis that occurs before birth and during the neonatal period is crucial for development of lung tissue and pulmonary function compatible with survival after birth in both human beings and other mammals (2).

Fatty acid synthesis requires NADPH as a cofactor, and it is generally accepted that NADPH is generated via the hexose monophosphate shunt. However, this cofactor is also produced in the reaction catalyzed by malic enzyme.

Malic enzyme (EC 1.1.1.40), which has been shown to be present in both cytosolic and mitochondrial fractions in most tissues (4-6) converts malate to pyruvate producing CO_2 and generating NADPH as shown in the following formula:



In addition to its role in providing NADPH, malic enzyme has

Received June 1, 1993; accepted December 13, 1993.

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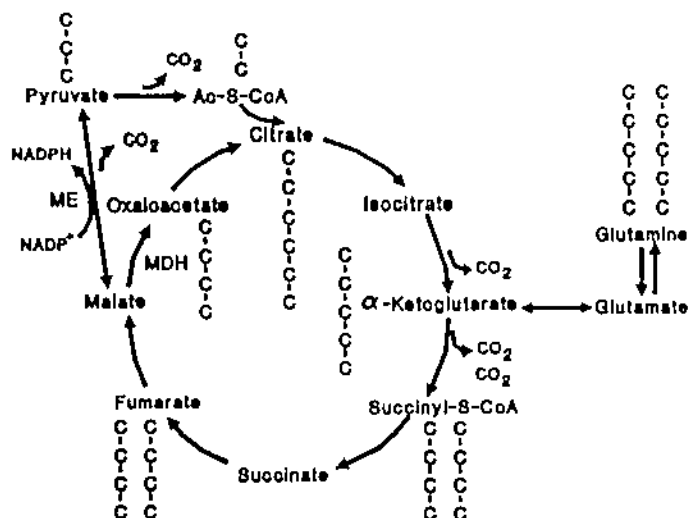


Fig. 1. The malate oxidation shunt. This pathway allows for the complete oxidation of glutamine and other anaplerotic substrates in the tricarboxylic acid cycle, allowing for the conversion of malate to pyruvate via malic enzyme (ME). In addition, NADPH is produced. MDH, malate dehydrogenase; Ac-S-CoA, acetyl CoA.

bovine serum were obtained from Paragon Biotechnology, Inc. (Baltimore, MD).

Tissue Preparation. Adult rat lung tissue. The animal protocols in this study were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (Zivic Miller, Zelenople, PA) weighing between 225–250 g and fed standard rat chow *ad libitum* were given heparin for anticoagulation and ketamine for anesthesia. They were exsanguinated, and the heart and pulmonary circulation were perfused with 50 mL of solution II (140 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4 , 10 mM [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 2.0 mM CaCl_2 and 1.3 mM MgSO_4 , pH 7.4) to remove blood from the lungs. The lungs were removed, and the large visible bronchi were excised. The lung tissue was placed in isolation medium (containing 0.25 mM sucrose, 0.5 mM EDTA, 100 mM Tris, pH 7.4) and minced on ice. The tissue was homogenized by hand with 10 strokes of an ice-jacketed Tenbroeck homogenizer (Wheaton Science Products, Inc., Millville, NJ). The homogenate was centrifuged at $6000 \times g$ to remove cell debris. The supernatant was recentrifuged at $34\,000 \times g$ to separate cytosolic and mitochondrial fractions. The mitochondrial pellet was resuspended in isolation medium and sonicated for 10 s three times in a Branson Sonifier to disrupt the mitochondrial membrane.

The presence of microsomes in the cytosolic fraction had no effect on the level of malic enzyme activity. When the cytosolic fraction was further centrifuged at $488\,000 \times g$ for 20 min to remove the microsomes, the malic enzyme activity was the same as the activity of the cytosolic fraction prepared as described above (data not shown). The mitochondrial/cytosolic ratio of lactate dehydrogenase was 0.17, which is consistent with lactate dehydrogenase being a predominately cytosolic enzyme. Succinate dehydrogenase, a predominately mitochondrial enzyme, had a mitochondrial/cytosolic ratio of 4.86.

Newborn rat lung tissue. Lungs from 1-d-old rat pups were obtained after decapitation. The pups, weighing an average of 8 g, had been kept with their dams and had been allowed to nurse freely. The dams were provided free access to standard rat chow and water. Lungs from two litters were combined to provide sufficient tissue. The tissue was minced and prepared as described above for the adult to obtain cytosolic and mitochondrial fractions.

L2 cells. L2 cells, cloned adult rat type II pneumocyte cells, were obtained from American Tissue Culture Collection (Rockville, MD). They were plated and cultured in minimum essential

medium with 10% fetal bovine serum for 7–10 days until grown to confluence in 95% air, 5% CO_2 at 37°C . The cells were washed twice with isolation media, scraped, pooled, and homogenized. The subcellular fractions were prepared as described above.

Measurement of Malic Enzyme Activity. Malic enzyme activity: Malic enzyme was assayed by measuring production of $^{14}\text{CO}_2$ from 1-[U- ^{14}C] malate. The assay is an adaptation of the method outlined by Hsu and Lardy (9) with the exception that $^{14}\text{CO}_2$ production is measured rather than NADPH production. Preliminary experiments revealed that the assay was proportional to protein concentration and gave results comparable to the spectrophotometric method. However, the sensitivity of the assay was increased about 10-fold compared with the spectrophotometric method, allowing for the use of small amounts of cells per tissue.

For these experiments the reaction mixture contained 75 mM Tris HCl, 4 mM NADP, 5 mM MnCl_2 , and varying concentrations of malate. Samples were incubated in a shaking water bath at 37°C . The reaction was started by the addition of the two subcellular fractions. Blanks with no tissue were run with each assay to correct for any nonenzymatic $^{14}\text{CO}_2$ release. At different times as described in the experimental protocol, the reaction was stopped with 0.3 mL of 10% (vol/vol) trichloroacetic acid. The rate of CO_2 production was measured by trapping the $^{14}\text{CO}_2$ in center wells containing methylbenzoniun hydroxide. The center wells were transferred to vials, and scintillation fluid was added and counted in a liquid scintillation spectrometer.

Protein concentration. Protein concentration of the tissue samples was determined by the Pierce BCA protein assay microreagent system (10). Enzyme activity was expressed in nmol/mg protein/min.

Statistical analysis. Each experiment was replicated at least three to four times. Kinetic parameters were determined with linear regression analysis. Data for the comparison of malic enzyme activity in adult and 1-d-old rat lung tissue were tested for significance using a paired *t* test.

RESULTS

The time course for malic enzyme activity as measured by $^{14}\text{CO}_2$ production is shown in Table 1 for both the cytosolic and mitochondrial fractions. During this 30-min time period, the rate was essentially linear from 15 to 30 min, and we chose time periods on that part of the curve for our subsequent kinetic and pH studies. The activity was measured in the presence and absence of NADP. The activity in cytosolic fraction in the absence of NADP was only $13.7 \pm 4.0\%$ (mean \pm SD) of the activity found when exogenous NADP was added. In contrast, in the absence of exogenous NADP, the mitochondrial fraction had approximately 50% the activity found when NADP was added to the reaction mixture ($50.3 \pm 6.5\%$, mean \pm SD),

Table 1. Effect of varying incubation time on $^{14}\text{CO}_2$ production (nmol/mg protein/min) at 2 mM malate concentration in both cytosolic and mitochondrial fractions of adult rat lung tissue, pH 7.4*

Time (min)	Cytosol		Mitochondria	
	With NADP	Without NADP	With NADP	Without NADP
5	2.16 \pm 0.23†	0.45 \pm 0.05	6.59 \pm 0.40	5.84 \pm 1.43
10	2.48 \pm 0.88	0.31 \pm 0.14	5.78 \pm 1.92	2.36 \pm 0.95
15	1.24 \pm 0.14	0.22 \pm 0.05	3.89 \pm 0.80	1.76 \pm 0.49
20	1.72 \pm 0.26	0.24 \pm 0.04	4.73 \pm 0.85	2.26 \pm 0.33
25	1.63 \pm 0.24	0.23 \pm 0.05	3.95 \pm 0.45	2.06 \pm 0.28
30	1.92 \pm 0.54	0.15 \pm 0.05	4.12 \pm 1.28	2.16 \pm 0.65

* Each time point was repeated three to five times and performed in quadruplicate during each experiment.

† Values are mean \pm SEM.

suggesting significant levels of endogenous NADP in the mitochondrial fraction.

To further characterize the enzyme in lung tissue, we examined the effects of pH on malic enzyme activity in adult rat lung tissue (Fig. 2). Varying the pH of the reactions had a differential effect on malic enzyme activity in the two subcellular fractions. When measured at increasing pH values, the cytosolic activity increased from a mean of 1.59 nmol/mg protein/min at pH 6.5 to a maximum of 3.96 nmol/mg protein/min at pH 8. The activity was about half that value at pH 9 (1.86 nmol/mg protein/min). In contrast, the enzyme activity in the mitochondria was essentially the same from pH 7 to 8 (3.07 nmol/mg protein/min at pH 7 and 2.52 nmol/mg protein/min at pH 8) but decreased to essentially zero at pH 8.5 and 9.

The effect of varying the initial substrate concentration on the enzyme activity in adult lung tissues in both cytosolic and mitochondrial fractions is shown in Figure 3. In the mitochondrial fraction, the enzymatic activity did not follow simple Michaelis-Menten kinetics as revealed by the Eadie-Hofstee plot (Fig. 3A) but was biphasic. This fraction showed an increase of activity throughout the 200-fold range of malate concentration. The Eadie-Hofstee plot shown in Figure 3A is best represented by two linear segments. Analysis of these data yielded a high-affinity K_m of 0.16 mmol, $V_{max} = 7.11$ nmol/mg protein/min, and a low-affinity K_m of 6.95 mmol, $V_{max} = 31.82$ nmol/mg protein/min.

In contrast to the mitochondria, the cytosolic enzymatic activity followed simple Michaelis-Menten kinetics, with a single K_m of 0.30 mmol, V_{max} of 5.95 nmol/mg protein/min, as shown in Figure 3B.

In paired experiments examining differences in enzyme activity between adult and 1-d-old rat lung tissue, malic enzyme was present in both mitochondrial and cytosolic fractions at both ages (Fig. 4). The activity was higher in the newborn, with a total mean activity of 11.16 nmol/mg protein/min as compared with mean activity in the adult lung of 8.07 nmol/mg protein/min ($p < 0.5$ paired t test). The mitochondrial activity in the newborn was 8.47 nmol/mg protein/min, which was more than twice the mitochondrial activity in the adult (3.92 nmol/mg protein/min) ($p < 0.05$, paired t test). In the adult cytosolic fraction the mean activity was 4.15 nmol/mg protein/min, whereas in the newborn

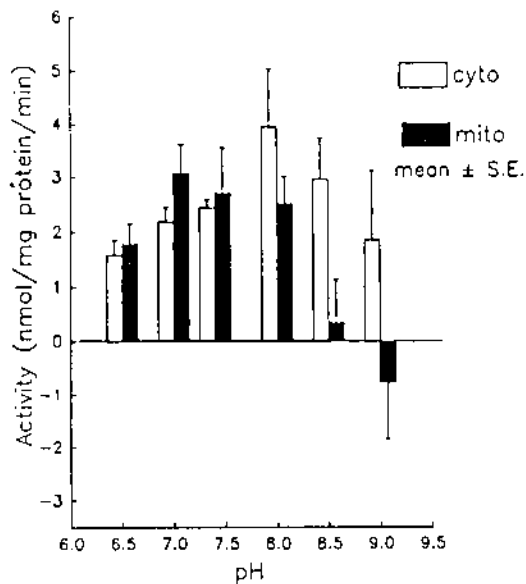


Fig. 2. The effect of pH of malic enzyme activity in adult rat lung tissue in both cytosolic (open bars) and mitochondrial (solid bars) fractions. The rate of $^{14}\text{CO}_2$ production was measured at pH 6.5, 7.0, 7.4, 8.0, 8.5, and 9.0 for 15-min incubation in the presence of 5 mM malate at 37°C as described in Materials and Methods. These results are mean \pm SEM from four experiments in which the activity at each pH value was measured in quadruplicate.

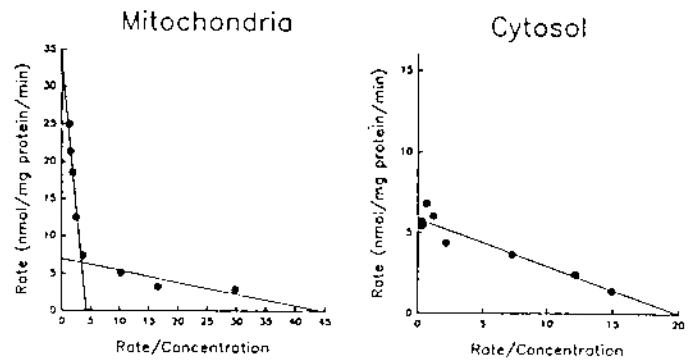


Fig. 3. Eadie-Hofstee plots of the effect of varying the initial substrate concentration on the malic enzyme activity in the mitochondria (A) and cytosol (B). Rate of $^{14}\text{CO}_2$ production was measured at malate concentrations of 0.1, 0.2, 0.5, 2.0, 5, 10, 15, and 20 mM during a 30-min incubation at 37°C, pH 7.4. Experiments were repeated three to six times at each concentration. Each concentration was done in quadruplicate during each experiment. The points represented are the mean values. A. The mitochondrial fraction kinetics are best represented by two linear segments of the Eadie-Hofstee plot: a high-affinity (0.1–2 mM) segment with a calculated K_m of 0.16 mM and V_{max} of 7.11 nmol/mg protein/min ($r^2 = 0.79$) and a low-affinity (2–20 mM) segment with a calculated K_m of 6.95, V_{max} of 31.82 nmol/mg protein/min ($r^2 = 0.96$). B. Kinetics in the cytosol where a single linear regression yielded a K_m of 0.30 mM and V_{max} of 5.95 nmol/mg protein/min ($r^2 = 0.90$) throughout the concentration range examined.

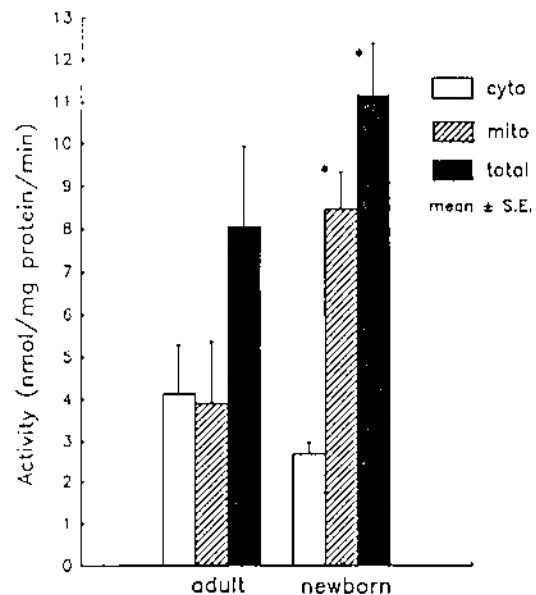


Fig. 4. Malic enzyme activity in adult and newborn rat lung tissue. In paired experiments examining differences in enzyme activity between adult and 1-d-old rat lung tissue, malic enzyme was present in both mitochondrial and cytosolic fractions at both ages. There was significantly higher total malic enzyme activity (solid bars) (*, $p < 0.05$) and significantly higher mitochondrial activity (hatched bars) in the newborn (*, $p < 0.05$) compared with the adult. This activity results in a shift of the ratio of mitochondrial to cytosolic activity from about 2.5 in the newborn rat lung to a ratio of about 1 in the adult. The cytosolic activity is shown in the open bars. Experimental conditions: pH 7.4, 37°C, 15-min incubation, 5 mM malate. Enzyme activity is expressed as nmol $^{14}\text{CO}_2$ produced/mg protein/min. Data shown as mean \pm SEM, $n = 4$. Within experiments, points were repeated in quadruplicate.

the mean activity was 2.69 nmol/mg protein/min. This activity results in a shift of the ratio of mitochondrial to cytosolic activity from about 2.5 in the newborn rat lung to a ratio of about 1 in the adult.

To further characterize this enzyme in the lung, we assayed the malic enzyme activity in cultured L2 cells. The cytosolic activity was 4.07 ± 2.47 nmol/mg protein/min (mean \pm SEM), and the mitochondrial activity was 1.98 ± 1.21 nmol/mg protein/min (mean \pm SEM) at 5 mM malate concentration and pH 7.4. The resultant ratio of mitochondrial to cytosolic activity was about 0.50 in these cells.

DISCUSSION

Using a sensitive technique developed in our laboratory, we were able to detect malic enzyme activity in both adult and newborn rat lung tissue and L2 cells, a cell culture line of type II pneumocytes. Previous attempts by other investigators to determine the presence of malic enzyme in lung tissue have produced varied results. Scholz and Rhoades (11) and Das and Ganguly (12) failed to detect malic enzyme activity in lung cytosol. Furthermore, these workers were not able to induce malic enzyme activity in the lungs of diabetic and hypophysectomized rats treated with triiodothyronine (12). Dozin *et al.* (13) reported malic enzyme activity as determined by rate of NADPH production in lung tissue. In addition, they found that malic enzyme mRNA was expressed in the lung. In their study (13), no change occurred in malic enzyme activity or amount of mRNA expressed after treatment with triiodothyronine in the lung, in contrast to the effect in the heart, liver, and kidney.

Batenburg and Whitsett (14) also measured malic enzyme mRNA and found no effect of nutritional status on the expression of malic enzyme mRNA in the lung. This finding was unlike the results in the liver, where starvation caused a small decrease and where refeeding produced a significant increase in mRNA. Although the changes in the expression of lung malic enzyme mRNA during starvation were not statistically significant, the results were suggestive of a decrease. This group did not measure enzyme activity, so if posttranscriptional changes in enzyme regulation had occurred, they would not have detected them.

In this report, we demonstrated that cytosolic and mitochondrial malic enzyme have several different properties. The mitochondrial fraction demonstrated biphasic kinetics within the 200-fold range of concentrations examined. However, in the cytosol, there was a single K_m and V_{max} . Although malic enzyme has been characterized in other tissues, including the brain and its cell types, astrocytes and synaptosomes (15, 16), no previous characterizations of the enzyme kinetics of malic enzyme in the lung have been made.

The enzymes in the two subcellular localizations also showed differences with respect to pH. The cytosolic enzyme demonstrated activity throughout the range of pH examined. The mitochondrial enzyme activity was more affected by pH, having the highest activity at pH values between 6 and 8. Our findings would argue that indeed two different enzymes are present in the cytosol and mitochondrial of the rat lung. This finding is in agreement with previous descriptions by Dozin *et al.* (13) of two different malic enzyme mRNAs in this tissue. This group has characterized the malic enzyme gene, and analysis of the 3' end demonstrated that use of alternate polyadenylation signals in exon 14 resulted in two mRNA with 3' untranslated regions of 345 and 1345 nucleotides, respectively (17).

Our results reveal important developmental differences in the activity of malic enzyme when the newborn rat lung was compared with adult lung. Significantly higher total malic enzyme activity, as well as significantly higher mitochondrial activity, occurred in the newborn. We did not control for dietary differences between the newborn and adult, which may have a role in the differences seen between newborn and adult malic enzyme activity. However, the higher rate of malic enzyme activity in the newborn suggests that alternate substrates such as anaplerotic amino acids (*e.g.* glutamine) may be used to a greater extent in

the young animal as energy substrates for cellular metabolism by way of the tricarboxylic acid cycle.

This concept is supported by our recent report of the higher use of glutamine when compared with glucose as an energy substrate by fetal rat lung fibroblasts (8). Because glutamine is the amino acid present in highest concentration in the serum (18), increased mitochondrial malic enzyme activity may play a key regulatory role to provide substrates for energy metabolism in the developing lung, as we have proposed (8). This mechanism may be particularly important in the newborn period when serum glucose levels may be low (19). Notably, glutamine has been shown to be a major energy substrate for several tissues, including brain (4) and intestine (20).

The role of ketone bodies in the neonatal period has been reviewed by Patterson and Rhoades (21). Yeh (22) and Sheehan and Yeh (23) have explored the incorporation of these substrates into lung phospholipids, showing that these substrates are important precursors for surfactant lipids in the neonatal animal. Furthermore, the enzymes involved in lipogenesis from ketone bodies are elevated in the newborn period in the lung (22), which correlates with our results of an elevated malic enzyme activity in the newborn lung. The involvement of multiple substrates for lipogenesis in the lung suggests the possible need for multiple sources of NADPH.

Yeh (24) demonstrated that type II cells isolated from newborn and adult rats incorporated ^{14}C -labeled acetoacetate into phosphatidylcholine, the major component of surfactant phospholipids. In the newborn rat, acetoacetate was used preferentially compared with glucose for the production of phosphatidylcholine. This description agrees with our findings in the L2 cells, where the ratio of cytosolic to mitochondrial malic enzyme activity showed a predominance of activity in the cytosol. This is the subcellular localization where activity would be expected if malic enzyme were used to generate NADPH for fatty acid synthesis used in the generation of surfactant phospholipids in the type II pneumocyte.

Indeed, the contribution malic enzyme makes to fatty acid synthesis can be estimated. In the adult, the cytoplasmic rate was approximately 4 nmol/mg protein/min. One mole of NADPH is produced for each mole of CO_2 produced. Fatty acid synthesis proceeding from acetyl CoA to palmitate requires 14 nmol NADPH for each nanomole of palmitate produced. In adult rat lung slices, Maniscalco *et al.* (25) determined the rate of fatty acid synthesis by measuring 3H incorporation from 3H_2O into total saponifiable fatty acids to be 0.28 nmol/mg protein/min. The NADPH required for this rate of fatty acid synthesis would be 3.92 nmol/mg protein/min. This finding would suggest that malic enzyme is capable of producing much of the NADPH necessary for fatty acid synthesis.

In summary, we have determined that malic enzyme is indeed present in both mitochondrial and cytosolic fractions of the rat lung. The kinetics of the enzyme in the two subcellular fractions were different, as was the behavior of the two isoenzymes at varying pH values. A significant increase occurred in both total malic enzyme activity and in the amount of malic enzyme present in the mitochondria in the newborn rat lung as compared with the adult, suggesting an important developmental role for the use of alternate substrates for energy metabolism in the lung.

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