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PHOSPHOPYRUVATE CARBOXYLASE OF RAT LUNG

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SUMMARY

Phosphopyruvate carboxylase [GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] activity was detected in adult rat lung and was associated predominantly with the mitochondrial subcellular fraction. The enzyme was more active with Mn^{2+} than Mg^{2+} and exhibited cofactor requirements similar to liver mitochondrial phosphopyruvate carboxylase. In the presence of Mn^{2+} the Michaelis constant for both lung and liver mitochondrial phosphopyruvate carboxylase was $7 \cdot 10^{-5}$ M. While liver non-particulate phosphopyruvate carboxylase was inhibited by malate in the presence of Mg^{2+}, both lung and liver mitochondrial enzymes were unaffected. Similarly, with Mg^{2+} as the bivalent cation only the lung and liver mitochondrial phosphopyruvate carboxylases were inhibited by AMP. The results indicate that the mitochondrial enzyme of rat lung has characteristics similar to those of the liver mitochondrial phosphopyruvate carboxylase.

INTRODUCTION

Phosphopyruvate carboxylase [GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32], an obligatory enzyme in gluconeogenesis in liver and kidney cortex, also has been reported in rat adipose tissue[1], brain[2], muscle[3,4] and lung[5,6]. With the possible exception of adipose tissue, the non-gluconeogenic function(s) of phosphopyruvate carboxylase in these other tissues remain obscure. In rat liver immunochemically distinct forms of this enzyme are found in the cell cytosol and mitochondria[7] and a number of characteristic kinetic differences have been reported between the cytosol and mitochondrial forms[8]. In addition, only the cytosol phosphopyruvate carboxylase has been shown to be affected by starvation or altered hormonal conditions[9,10]. While the subcellular distribution of this enzyme also has been studied in adipose tissue[1,11] and brain[2], little is known about the characteristics of this enzyme in rat lung.

A previous report[5] suggested the presence of phosphopyruvate carboxylase activity in non-particulate subcellular preparations of rat lung, although this finding was not considered conclusive. It was the purpose of the present investigation to

provide conclusive evidence for the presence of phosphopyruvate carboxylase in rat lung, its subcellular distribution, and to compare selected properties of the lung enzyme in relatively crude preparations with those characteristics of the cytosol and mitochondrial enzymes of the liver. It was considered that this information would be necessary before a generalized role of this enzyme could be assigned for lung tissue.

EXPERIMENTAL

Animals and tissue preparation

Male Long–Evans hooded rats averaging 250–350 g body weight were used in all experiments. All rats were housed individually in stainless steel cages with raised wire floors, fed on a diet of laboratory rat pellets (Purina Laboratory Chow) and had free access to water. Starved rats were deprived of food for 48 h. Temperature, relative humidity and light were controlled at 22 °C, 50% and a 12-h photoperiod, respectively.

Animals were anesthetized with sodium pentobarbital (Halatal solution, Jensen–Salsbery Laboratories, Kansas City, Mo., U.S.A.), 3 mg/100 g body weight intraperitoneally, and the lungs perfused with 0.85% NaCl *in situ* as described by Scholz and Rhoades[12]. Crude homogenates of lung and liver tissue were prepared as described previously[5] in 9 vol. of 0.25 M sucrose, pH 7.4, containing 10 mM Tris and 1 mM dithiothreitol.

Subcellular fractionation

Crude homogenates were twice centrifuged at 600 × *g* for 10 min and the resulting supernatant centrifuged at 14 000 × *g* for 15 min. The 14 000 × *g* supernatant was centrifuged at 105 000 × *g* for 1 h. The 105 000 × *g* pellet was washed with homogenizing buffer and resuspended in 0.05 M Tris, pH 8.0, and was considered the microsomal fraction. The 105 000 × *g* supernatant was considered the non-particulate fraction. The 14 000 × *g* pellet was washed and resuspended in homogenizing buffer and centrifuged again at 14 000 × *g* for 15 min. The final pellet was washed with homogenizing buffer, freeze-dried and resuspended in 0.05 M Tris, pH 8.0. This crude preparation was considered the mitochondrial fraction and used for studies conecrned with the subcellular distribution of phosphopyruvate carboxylase. The presence of mitochondria in this fraction was verified by electron microscopy, marker enzymes and polarographic assays of oxygen uptake and respiratory control.

For all subsequent experiments the initial steps in the purification procedure described by Ballard[8] were followed. Lung tissue from 3–5 rats was pooled and homogenized as described. The resuspended freeze-dried mitochondrial fraction was centrifuged at 30 000 × *g* for 20 min and the resulting supernatant brought to 0.65 saturation with a solution of saturated $(NH_4)_2SO_4$, pH 7.0. The solutions were allowed to stand on ice for 45 min and then centrifuged again at 30 000 × *g* for 30 min. The resulting precipitate was resuspended in a minimum volume of 0.75 saturated $(NH_4)_2SO_4$, pH 7.0, containing 0.5 mM GSH and 0.5 mM EDTA. The 105 000 × *g* supernatant was treated with $(NH_4)_2SO_4$ in an identical manner. It was found that the enzyme stored in this form at 2 °C was stable for at least one week.

Assay of enzyme activities

Phosphopyruvate carboxylase was routinely assayed by the method of Seubert and Huth[13]. Unless otherwise noted, the reaction mixture contained (in μmoles): Tris (pH 8.1), 100; GSH, 2.0; $MgSO_4$, 28; KCl, 30; NaF, 30; ITP, 12; and oxaloacetate, 9.0, in a total volume of 2.0 ml. Oxaloacetate solutions were always freshly prepared and neutralized prior to assay. Reactions were measured for 5 or 10 min at 37 °C and stopped with the addition of solid KBH_4. The excess KBH_4 was removed with 15% $HClO_4$ and the solutions neutralized with 3 M $KHCO_3$. The solutions were centrifuged and the phosphoenolypyruvate formed was determined in the clear supernatant as described by Czok and Eckert[14]. Preliminary studies showed that, under these conditions, added amounts of phosphoenolpyruvate to the assay system were essentially completely recovered.

For the estimation of the K_m for oxaloacetate, the Seubert and Huth[13] assay as modified by Ballard[8] was used. In this case the reaction mixture contained (in μmoles): imidazole (pH 7.0), 200; $MnCl_2$, 2.0; ITP, 1.6; GSH, 2.0; enzyme preparation containing approximately 0.1 mg protein; and oxaloacetate at the indicated amounts in a total volume of 2.0 ml. In a preliminary study, the non-enzymatic breakdown of oxaloacetate in this assay system was found to be significant as previously reported by Ballard[8], but independent of the initial oxaloacetate concentration (Fig. 1). In all

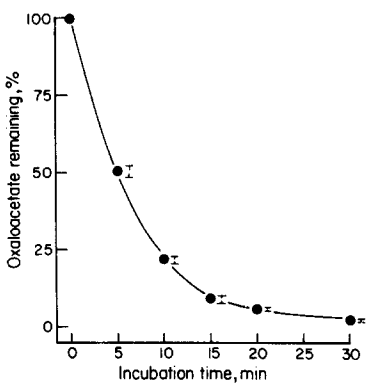

Fig. 1. Non-enzymatic breakdown of oxaloacetate in the presence of Mn^{2+}. Assays were conducted at 37 °C as described in the text, *minus* enzyme, with calculated oxaloacetate concentrations of (mM): 0.4, 0.2, 0.1, 0.05, 0.03 and 0.02. At zero time the assay medium oxaloacetate was directly determined and subsequent oxaloacetate levels are expressed as a percent of this value. Each point represents the mean ± S.E. of the percent oxaloacetate remaining at 6 different concentrations for the times indicated.

estimations of K_m the initial oxaloacetate concentration was determined by the method of Hohorst and Reim[15] and the effective concentration per 10-min assay interpreted from the data given in Fig. 1. Phosphoenolpyruvate recovery from this assay system was not complete due to non-enzymatic hydrolysis (Nordlie and Lardy[16]); however this did not prevent the determination of K_m for oxaloacetate.

Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) activity was measured by the method of Bucher and Pfleiderer[17]. The activity of enolase (phosphopyruvate hydratase, EC 4.2.1.11) was determined as described by Holt and Wold[18]. Citrate synthase [citrate:oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7] was as-

sayed by the method of Srere[19] and was used as a mitochondrial marker. 6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase, EC 1.1.1.44) was assayed by the method of Glock and McLean[20] and was used as a non-particulate fraction marker. Protein was determined by a modified biuret procedure[21].

RESULTS

The subcellular distributions of 6-phosphogluconate dehydrogenase, citrate synthase and phosphopyruvate carboxylase in fed and starved rat lung is shown in Fig. 2. As lung tissue homogenates contain large amounts of connective tissue the

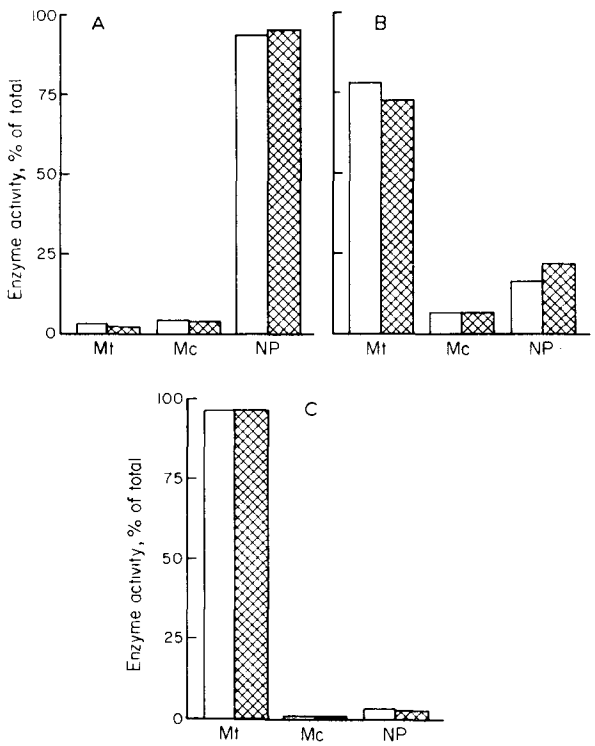

Fig. 2. Subcellular distribution of lung 6-phosphogluconate dehydrogenase (A), citrate synthase (B), and phosphopyruvate carboxylase (C) in fed (open bars) and starved (shaded bars) rats. Values represent the means of six determinations expressed as a percent of total activity. Subcellular fractions are designated Mt (mitochondrial), Mc (microsomal), and NP (non-particulate).

nuclei and cell debris fractions were not included in this study. Greater than 95% of the total activity of phosphopyruvate carboxylase was found in the mitochondrial fraction from both fed and starved rat lung. Fig. 3 shows similar data for rat liver. In essential agreement with previous reports[10,16,22], phosphopyruvate carboxylase in rat liver is predominantly a soluble enzyme. Greater percentages of total citrate synthase were detected in the non-particulate fraction of both lung and liver preparations of fasted rats as compared with fed rats.

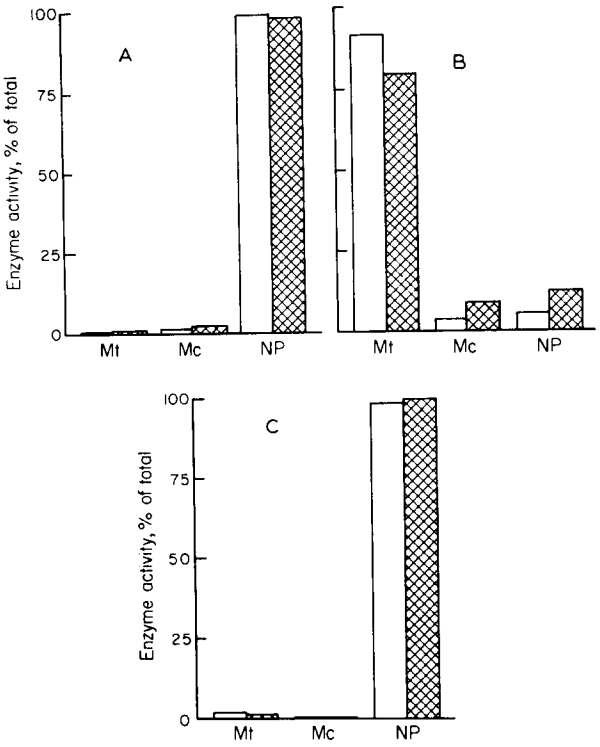

Fig. 3. Subcellular distribution of liver 6-phosphogluconate dehydrogenase (A), citrate synthase (B) and phosphopyruvate carboxylase (C) in fed (open bars) and starved (shaded bars) rats. Refer to Fig. 2 legend for additional details.

Table I shows that while the total activity of the soluble liver phosphopyruvate carboxylase was significantly increased ($P < 0.05$) during fasting, the lung enzyme was not affected. This response is similar to that demonstrated for the mitochondrial phosphopyruvate carboxylase in rat liver[10] but contrasts with that reported for rat lung cytosol preparations[6].

Fig. 4 shows phosphopyruvate carboxylase activity as a function of assay pH. Under the standard assay conditions the lung mitochondrial and liver soluble enzymes

TABLE I

EFFECT OF FASTING ON RAT LIVER AND LUNG PHOSPHOPYRUVATE CARBOXYLASE

The values are given as means ± S.E. of five observations each. One unit represents enzyme activity catalysing the production of 1 μmole of phosphoenolypyruvate/min per g of tissue at 37 °C.

Dietary status	*Enzyme source*	
	Liver non-particulate	*Lung mitochondria*
Fed *ad libitum*	6.61 ± 0.54	0.036 ± 0.003
Fasted 48 h	10.88 ± 0.96*	0.036 ± 0.005

* $P < 0.05$ as compared with the corresponding value for control rats fed *ad ilibitum*.

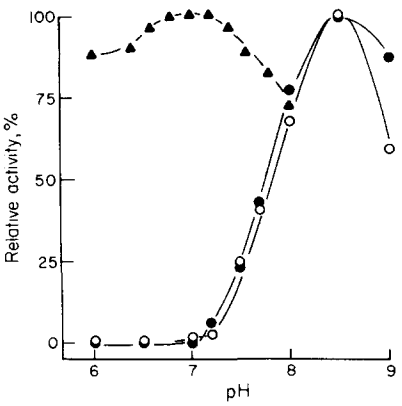

Fig. 4. Phosphopyruvate carboxylase activity as a function of assay pH. Liver non-particulate (○) and lung mitochondrial (●) enzyme assays were determined by the method of Seubert and Huth[13]. The activity of the lung mitochondrial enzyme (▲) was also assayed by the method of Seubert and Huth[13] as modified by Ballard[8].

had similar pH optima. The pH optimum of 8.1 for the liver and lung enzymes is similar to that reported by Holten and Nordlie[25] for liver phosphopyruvate carboxylase using similar assay conditions. The difference in the pH optima of the lung mitochondrial enzyme between the standard assay (Seubert and Huth[13]) and that modified by Ballard[8] is depicted only to demonstrate that the activity of this enzyme as a function of pH is directly related to the assay conditions and may be of little physiological importance.

The requirements for the liver and lung mitochondrial enzymes are compared in Table II. In general agreement with previous reports on the cofactor specificity of rat liver[22] and adipose tissue[26] phosphopyruvate carboxylases, both liver and lung enzymes exhibited more activity with ITP than GTP, and slight activity with UTP,

TABLE II

REQUIREMENTS FOR RAT LIVER AND LUNG MITOCHONDRIAL PHOSPHOPYRUVATE CARBOXYLASE

Activity is expressed as nmoles phosphoenol pyruvate formed/min per mg of protein at 37 °C. The complete system contained (in μmoles): Tris, pH 8.1, 100; GSH, 2; $MgSO_4$, 28; KCl, 30; NaF, 30; ITP, 12; oxaloacetate, 9; and enzyme preparation containing approximately 0.1 mg protein in a total volume of 2.0 ml. Freeze-dried mitochondrial preparations were treated as described in the methods section.

Additions or deletions to complete system	*Enzyme activity*	
	Liver	*Lung*
Complete system	6.8	12.5
−ITP, +GTP	5.6	8.9
−ITP, +UTP	1.4	1.7
−ITP, +ATP	0	0
−ITP, +CTP	0	0
−ITP	0	0
−Oxaloacetate	0	0
$-Mg^{2+}$	0	0
$-Mg^{2+}$, $+Mn^{2+}$	12.4	27.7
−Enzyme, + boiled enzyme	0	0

while ATP and CTP proved ineffective. Kurahashi *et al.*[27] observed a similar slight activity of avian mitochondrial phosphopyruvate carboxylase in the presence of UTP which they attributed to chromatographically inseparable ITP or GTP contamination of the UTP used. In agreement with the findings of Chang and Lane[28], when IDP replaces ITP in the assay system, pyruvate is produced by both the lung and liver mitochondrial enzymes, although at a reduced rate as compared with phosphoenolpyruvate synthesis in the presence of ITP (data not shown). Mn^{2+} was a more effective activator than Mg^{2+} for both lung and liver enzymes.

The effect of two concentrations of AMP, malate, and oxalate on the activities of liver and lung phosphopyruvate carboxylase in the presence of Mg^{2+} or Mn^{2+} is shown in Table III. In the presence of Mg^{2+}, both lung and liver mitochondrial enzymes were inhibited by 2 mM AMP while the liver non-particulate enzyme was not. While all enzymes were inhibited by 2 mM AMP in the presence of Mn^{2+}, only the mitochondrial enzymes were inhibited by 0.5 mM AMP when either Mn^{2+} or Mg^{2+} served as bivalent cation. Malate inhibition could not be shown for either the lung or liver mitochondrial enzymes; however the liver non-particulate enzyme was inhibited by 40 mM malate in the presence of Mg^{2+}. Oxalate, as previously reported for the liver enzyme[13], proved to be a potent inhibitor of lung phosphopyruvate carboxylase.

TABLE III

INHIBITION BY AMP, MALATE, AND OXALATE OF RAT LIVER AND LUNG PHOSPHOPYRUVATE CARBOXYLASE

Activity was determined by the method of Seubert and Huth[13] and expressed as a percent of activity with Mn^{2+} or Mg^{2+} in the absence of inhibitor.

Inhibitor	*Enzyme activity (%)*					
	Lung mitochondrial		*Liver non-particulate*		*Liver mitochondrial*	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
2 mM AMP	73	67	96	72	70	78
0.5 mM AMP	68	80	97	99	78	85
40 mM malate	93	101	71	100	99	99
2 mM malate	99	97	88	100	102	97
2 mM oxalate	0	0	0	0	0	0
0.5 mM oxalate	8	3	3	3	3	4

Ballard[8] found a consistent difference between mitochondrial and soluble phosphopyruvate carboxylases with respect to the K_m for oxaloacetate in the presence of Mn^{2+}. In the present study, without a highly purified enzyme preparation, the determination of phosphopyruvate carboxylase activity at very low oxaloacetate concentrations was not possible for liver non-particulate preparations using the Ballard[8] assay due to considerable contamination with pyruvate kinase and enolase activities. The mitochondrial $(NH_4)_2SO_4$ precipitated preparations from lung and liver, however, were found to be free of measurable pyruvate kinase and enolase activities. Furthermore, no detectable malate formation was observed in this assay system, even at high oxaloacetate concentrations (4.5 mM). This preparation contained malate dehydrogenase activity; however malate formation occurred only when an exogenous source of NADH was added to the assay. Fig. 5 shows the double

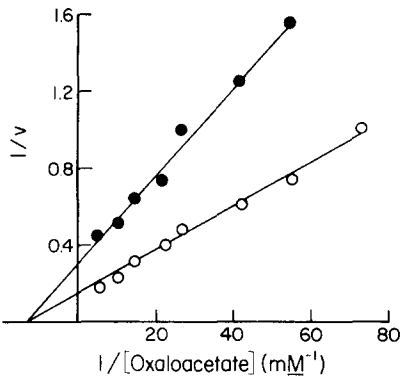

Fig. 5. Double reciprocal plot of velocity (nmoles phosphoenolpyruvate/min) against oxaloacetate concentration (mM) in the presence of 1 mM Mn^{2+} for lung (●) and liver (○) mitochondrial phosphopyruvate carboxylase. Activity was determined by the method of Seubert and Huth[13] as modified by Ballard[8] at pH 7.0. Refer to text for further details.

reciprocal plots for the estimation of K_m for oxaloacetate in the presence of Mn^{2+} for lung and liver mitochondrial preparations. From the data shown in Fig. 5 the estimated K_m for oxaloacetate was $7 \cdot 10^{-5}$ M for both the liver and lung mitochondrial enzymes. In this assay system Mg^{2+} was not an effective activator of either the lung or liver mitochondrial enzyme, consequently the apparent K_m for oxaloacetate in the presence of Mg^{2+} was not determined.

DISCUSSION

While phosphopyruvate carboxylase is found in the cytosol of rat adipose tissue[1], it is apparently a mitochondrial enzyme in brain tissue[2]. The results of the present experiments with rat lung subcellular preparations indicated that the mitochondrial fraction contained most of the phosphopyruvate carboxylase activity in this tissue. It was observed that a greater percentage of the total citrate synthase activity was recovered in the extramitochondrial fractions of lung and liver preparations of the starved animals. Although citrate synthase is believed to be exclusively a mitochondrial enzyme[23], this probably reflects increased mitochondrial breakage in the starved condition. In this context, Taylor *et al.*[24] recently presented evidence of increased mitochondrial fragility in the starved sheep liver as compared with the fed condition in their studies concerned with the subcellular distribution of phosphopyruvate carboxylase and pyruvate carboxylase. The greater proportion of citrate synthase activity appearing in the lung non-particulate fraction compared with that for the liver in the present studies may be a reflection of the more severe homogenization technique required in the preparation of lung homogenates and is an indication of more extensive mitochondrial breakage. This suggests that most of the phosphopyruvate carboxylase activity found in lung non-particulate fractions is a result of mitochondrial breakage and that phosphopyruvate carboxylase in the lung is essentially a mitochondrial enzyme. These data, however, do not exclude the possibility that part of the observed enzyme activity also is associated with lysosomes, or some other particulate subcellular element that sediments with the mitochondria. From the results of the present studies it appears that much of the phosphopyruvate

carboxylase activity previously reported for rat lung cytosol preparations[5] resulted from mitochondrial release. Hanson and Garber[6] have also recently reported phosphopyruvate carboxylase activity in cytosol preparations of rat lung; however, similar data for subcellular fractions other than the cell cytosol were not presented.

Differences have been shown in the catalytic properties of the cytosol and mitochondrial enzymes in liver, and these differences apparently also extend to the mitochondrial enzyme of rat lung. One of the objectives of the present study was to compare known characteristic differences of the mitochondrial and non-particulate phosphopyruvate carboxylases of rat liver with those of the lung mitochondrial enzyme. Holten and Nordlie[25] found that only the mitochondrial enzyme of liver was inhibited by 1.33 mM AMP in the presence of Mg^{2+}. In the present study, a similar inhibition by 2 mM AMP was observed for the mitochondrial enzyme of lung. Ballard[8] reported that at high oxaloacetate concentrations (1 mM), only the cytosol enzyme of liver was inhibited by 40 mM malate at pH 7.0 in the presence of Mg^{2+}. In this context, we observed that at pH 8.1 and 4.5 mM oxaloacetate, only the non-particulate liver enzyme was inhibited by malate in the presence of Mg^{2+}, while both the lung and liver mitochondrial enzymes were not affected.

A consistent difference was observed by Ballard[8] between the mitochondrial and soluble liver phosphopyruvate carboxylases with respect to the K_m for oxaloacetate in the presence of Mn^{2+}. The K_m for oxaloacetate of $7 \cdot 10^{-5}$ M is substantially higher than the $9 \cdot 10^{-6}$ M reported by Ballard[8] for chicken and sheep liver mitochondrial enzymes under similar assay conditions. Although the enzyme preparations in the present study were not highly purified, linear double reciprocal plots were obtained, and the apparent K_m values of both the lung and liver mitochondrial enzymes were similar with Mn^{2+} as the divalent cation.

The observation of Holten and Nordlie[25], that the liver mitochondrial enzyme exhibits greater activity with Mg^{2+} than Mn^{2+} at pH 8.1, could not be confirmed in the present study. Regardless of the enzyme source, Mn^{2+} was a more effective activator than Mg^{2+} under all conditions tested. While there is no apparent explanation for this discrepancy, Holten and Nordlie[25] used purified enzyme preparations and perhaps this is a necessary step for further study of the lung mitochondrial enzyme.

It is well established that mammalian lung actively participates in the synthesis of lipids, particularly phospholipids, which are important constituents of the pulmonary surfactant system. The functional importance of a mitochondrial phosphopyruvate carboxylase is not clear at present. In tissues actively participating in gluconeogenesis, *viz.*, liver and kidney cortex, this enzyme catalyzes a key reaction in the reversal of glycolysis. It has been suggested that phosphopyruvate carboxylase in non-gluconeogenic adipose tissue plays a role in the formation of glyceride–glycerol, particularly when the availability of glucose is limited[1,11,29]. Whether a similar glyceroneogenic role can be assigned to mammalian lung is only speculative at this time but may in part account for the observed low-level incorporation of [^{14}C]pyruvate into lung lecithin glycerol[30]. Additional functional roles of phosphopyruvate carboxylase in lung tissue possibly could be associated with the formation of mitochondrial oxaloacetate, *via* CO_2 fixation, or with a coupling to the substrate level phosphorylation of α-oxoglutarate. It is anticipated that additional studies with isolated lung mitochondrial preparations could provide more information with

respect to metabolic function at these levels. Although not necessarily related to phosphopyruvate carboxylase, the high specific activity of mammalian lung guanyl cyclase[31,32] and guanosine 3′,5′-monophosphate phosphodiesterase[33] may be associated with an active metabolism of guanine nucleotides in this tissue.

It is emphasized that the total activity of lung phosphopyruvate carboxylase is very low when compared with that for the soluble enzyme of rat liver. However liver is a comparatively homogeneous tissue whereas mammalian lung is composed of a number of morphologically distinct cell types, each with divergent metabolic activities[34]. It is conceivable that the activity of the lung enzyme, expressed on a whole tissue basis, may be misleading if the enzyme is associated with a specific cell type.

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