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PHOSPHOENOLPYRUVATE CARBOXYLASE DECARBOXYLATION CATALYZED REACTION IN CYTOSOL OF RAT ADIPOSE TISSUE

O. MEYUHAS, CH. BOSHWITZ AND L. RESHEF
Department of Biochemistry, The Hebrew University, Hadassah Medical School, Jerusalem (Israel)

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SUMMARY

The activity of phosphoenolpyruvate carboxylase (ITP:oxaloacetate carboxylyase (transphosphorylating)) in crude extracts of adipose tissue and liver was measured in the direction of phosphoenolpyruvate formation. Knowledge of the maximal rate of this process under these conditions is of importance in the evaluation of the physiological significance of changes in enzyme level induced by various treatments. The substrate (oxaloacetate) may be added either directly or generated (a) from malate catalyzed by malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) in the presence of NAD^+ and pyruvate, (b) from aspartate and α-ketoglutarate, catalyzed by aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1, formerly known as glutamate:oxaloacetate transaminase). All these yielded a similar maximal activity. With the adipose tissue enzyme, the K_m value for oxaloacetate, whether supplied directly or generated was $2.2 \cdot 10^{-5}$ M. With liver enzyme a similar K_m value was obtained only if oxaloacetate was generated, while a higher value ($3 \cdot 10^{-4}$ M) was found by directly adding oxaloacetate.

Both oxaloacetate and malate, at high concentrations inhibit phosphoenolpyruvate carboxylase activity. The inhibition of the adipose tissue enzyme, but not the liver enzyme, may be overcome by the addition of pyruvate. In the presence of pyruvate, v_{max} for phosphoenolpyruvate formation was attained with $3 \cdot 10^{-3}$ M malate, as a source of oxaloacetate. Half maximal activity was found with $2.3 \cdot 10^{-4}$ M malate. This concentration of malate is physiological and therefore implies that the activity of the enzyme *in vivo* rarely exceeds its half maximal activity.

INTRODUCTION

The activity of the phosphoenolpyruvate carboxylase (ITP:oxaloacetate carboxy-lyase (transphosphorylating)) in crude tissue extracts is determined by following either the rate of oxaloacetate decarboxylation[1] or the carboxylation of phosphoenolpyruvate[2] as outlined by the following reaction:

$$\text{Oxaloacetate} + \text{ITP} \rightleftharpoons \text{phosphoenolpyruvate} + \text{IDP} + CO_2$$

Both of these reactions were described by UTTER AND KURAHASHI[3,4]. The oxaloacetate decarboxylation reaction was employed by NORDLIE AND LARDY[1] and involved a chemical determination of phosphoenolpyruvate formed by the ITP dependent decarboxylation of oxaloacetate. This method proved not to be sensitive enough as it failed to detect low enzyme activities like those found in adipose tissue[5]. On the other hand, these low enzyme activities could be measured by the carboxylation reaction which determined the rate of $NaH^{14}CO_3$ fixation in the presence of ITP, Mn^{2+} and phosphoenolpyruvate[2].

The use of this method revealed that the activity of adipose tissue phosphoenolpyruvate carboxylase is subject to remarkable changes induced by a variety of treatments[2,6–8]. These changes were closely co-related with those found in the rate of the glyceride glycerol synthesis from pyruvate[8]. Thus, a role for this enzyme in the regulation of the glyceroneogenic activity was indicated. To substantiate this indication and to show that this enzyme may be rate limiting in the glycerogenesis from pyruvate, the activity of the enzyme in the direction of glycerogenesis, *i.e.* decarboxylation, has to be established. However, the enzyme activity in adipose tissue has so far been assayed only by the carboxylation method and the rate of the decarboxylation catalyzed reaction is not known. Recently, CHANG *et al.*[9] who studied the kinetics of a purified mitochondrial enzyme preparation from pig liver, have shown that the rate of the oxaloacetate decarboxylation was 10 times faster than the rate of phosphoenolpyruvate carboxylation. If this ratio also holds for the adipose tissue enzyme, the rate of the decarboxylation reaction would exceed the rate of the glyceride glycerol synthesis by more than an order of magnitude, and it would be impossible to maintain the supposition that phosphoenolpyruvate carboxylase indeed is a key enzyme in the synthesis of the glyceride glycerol.

Consequently, it became important to obtain direct measurements of the decarboxylation activity in crude extracts of adipose tissue, and to quantitate the amount of the enzyme in this way.

EXPERIMENTAL PROCEDURE

Animals

Male albino rats from the Jerusalem Hebrew University breeding center (Wistar Origin) were fed on a local diet with a composition similar to the purina chow. Adrenalectomized rats were maintained on 1% NaCl in the drinking water and used on the 5–10th post-operative days. The rats used throughout the experiments were adrenalectomized and fasted for 24 h unless indicated otherwise in the text.

Materials

Malate, phosphoenolpyruvate, ITP, IDP, GTP, CTP, UTP, NAD^+, NADH, L-aspartate and α-ketoglutarate were all Sigma preparations and used without further purification. NAD^+–malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) (specific activity 820 units/mg protein) was from Sigma. Lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) free of pyruvate kinase activity (specific activity of 360 units/mg protein) and pyruvate kinase (ATP:pyruvate phos-

photransferase, EC 2.7.1.40) (specific activity of 150 units/mg protein) were purchased from Boehringer and Sohne. Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1 formerly known as glutamate:oxaloacetate transaminase) was prepared from rat hearts. Supernatants obtained by centrifugation at 100 000 × *g* for 30 min were used without further purification. $NaH^{14}CO_3$ was from Radiochemical Centre, Amersham, England.

Enzyme assay

Adipose tissue was homogenized in 4 vol. of buffered sucrose[10] and liver in 9 vol. of the same solution. Supernatants obtained by centrifugation at 100 000 × *g* were used for enzyme assays.

Phosphoenolpyruvate carboxylase assay method

Carboxylation assay. This was performed essentially according to CHANG AND LANE[11] as adapted for crude adipose tissue extracts by BALLARD *et al.*[2]. The fixation of ^{14}C labeled $NaH^{14}CO_3$ was measured in the presence of phosphoenolpyruvate, IDP, $MnCl_2$, dithiothreitol, NADH, malate dehydrogenase and tissue extract. The following concentrations were included in the reaction mixture: Imidazole buffer, pH 6.6, 100 mM; phosphoenolpyruvate, 15 mM; $MnCl_2$, 20 mM; IDP, 1.25 mM; $KHCO_3$, 50 mM (containing 2 μC of $NaH^{14}CO_3$); dithiothreitol, 1 mM; NADH, 2.5 mM; 2 units of malate dehydrogenase; and tissue extract in a total volume of 1.0 ml. The assay was carried out for 10 and 20 min, and the reaction was stopped by addition of trichloroacetic acid to a final concentration of 3.3%. After each tube was gassed with CO_2 for 5 min, the radioactivity was determined with Diotol as solvent[12] in a Packard tricarb liquid scintillation spectrometer. A unit of enzyme activity is defined as 1 μmole of bicarbonate fixed per min, at 37°.

Decarboxylation assay. (a) Oxaloacetate added directly. The ITP and Mn^{2+} dependent decarboxylation of oxaloacetate was measured by the determination of phosphoenolpyruvate formed at the end of the reaction. The assay reaction mixture contained the following components at their final concentrations. Tris–HCl buffer, pH 8, 50 mM; $MnCl_2$, 0.75 mM; ITP, 0.75 mM; oxaloacetate, 0.15 mM; and tissue extract in a total volume of 1.0 ml. The reaction mixture was initiated by the addition of freshly dissolved oxaloacetate. At the end of 4 min and 2 min at 37° for adipose tissue and liver supernatants respectively, the content of each tube was completely transferred to a tube containing 0.9 ml of boiling water in a boiling water bath. Following 5 min boiling, the tubes were cooled in an ice bath, centrifuged and phosphoenolpyruvate determined by the method of CZOK AND ECKERT[13]. Prior to the addition of pyruvate kinase, lactate dehydrogenase NADH and ADP were added to each tube for the removal of pyruvate. Then, the amount of phosphoenolpyruvate was determined by the amount of NADH oxidized upon the addition of pyruvate kinase. 1 unit of enzyme activity is the amount of tissue supernatant which will catalyze the ITP dependent formation of 1 μmole of phosphoenolpyruvate per min at 37°.

(b) Oxaloacetate generated from malate. In assay systems where oxaloacetate was generated from malate, the generating system contained the following components in their final concentrations. Tris–HCl buffer, pH 8, 50 mM; malate dehydrogenase 1–2 units (not necessary to add); NAD^+, 1 mM; $MnCl_2$, 0.75 mM; and malate, 20 mM;

ITP, 0.75 mM. The reaction mixture was brought to an equilibrium by incubation for 3 min at 37° followed by the addition of tissue supernatant which was equilibrated for another min. Then ITP was added to initiate phosphoenolpyruvate carboxylase activity. Henceforth the procedure was as outlined for oxaloacetate.

(c) Oxaloacetate generated from aspartate. In this case, similar to the previous generation system, an equilibrium of the system was first attained by the addition of aspartate, α-ketoglutarate and aspartate aminotransferase =prerequisite for maximal activity) and incubation for 3 min at 37° followed by the addition of tissue supernatant for another min. Then phosphoenolpyruvate carboxylase activity was initiated by ITP. The complete assay system in total volume of 1 ml contained the following components in their final concentration: Tris–HCl buffer, 50 mM; L-aspartate, 1 mM; α-ketoglutarate, 1 mM; $MnCl_2$, 0.75 mM; aspartate aminotransferase, 0.5 unit; ITP, 0.75 mM; and tissue supernatant. At the end of the reaction, phosphoenolpyruvate was determined as previously described.

Aspartate aminotransferase activity

This was determined spectrophotometrically according to BERGMEYER AND BERNT[14].

Equilibrium constants

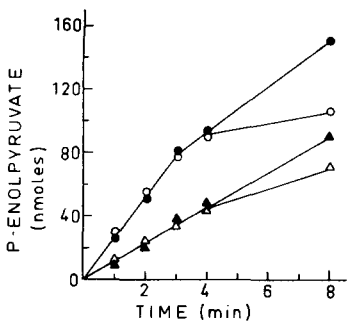
The equilibrium constant used for the malate dehydrogenase system was taken from STERN *et al.*[15].

TABLE I

REQUIREMENTS FOR PHOSPHOENOLPYRUVATE CAROBOXYLASE DECARBOXYLATION CATALYZED REACTION

Adipose tissue and liver extracts were used as enzyme preparations. The reaction mixture contained the following components in their final concentration: 50mM Tris–HCl buffer, pH 8.0; 0.75 mM ITP; 0.75 mM $MnCl_2$; 0.15 mM. Oxaloacetic acid (freshly dissolved) and enzyme (0.05 ml of adipose tissue and 0.02 ml of liver supernatants) in total volume of 1 ml. Similar concentrations of other nucleotides (0.75 mM) or $MgCl_2$ concentration as indicated in the table were added when indicated. The reaction was initiated by the addition of oxaloacetate, carried out at 37° for 2 min with liver enzyme and 4 min with adipose tissue enzyme, and terminated by boiling. Phosphoenolpyruvate was determined as described under EXPERIMETNAL PROCEDURE.

Addition to or deletion from assay reaction mixture	*Phosphoenolpyruvate formation (nmoles/min)*		
	Adipose tissue	*Liver*	
		a	*b*
None (complete)	9	48	44
Tissue extract deleted	1	1	
ITP deleted	2	3	3
$MnCl_2$ deleted	1	1	
Oxaloacetate deleted	2	4	
ITP deleted, GTP added	5	28	
ITP deleted, UTP added	3	18	
ITP deleted, ATP added	2	6	
ITP deleted, CTP added	2	8	
$MnCl_2$ deleted, 0.75 μmole $MgCl_2$ added			22
$MnCl_2$ deleted, 1.0 μmole $MgCl_2$ added			19
$MnCl_2$ deleted, 1.5 μmoles $MgCl_2$ added			19



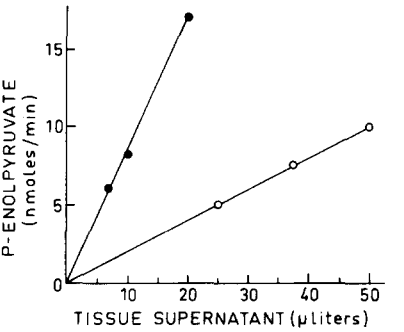


Fig. 1. Time course of the ITP-dependent phosphoenolpyruvate formation catalyzed by liver (○, ●) and adipose tissue supernatants (△, ▲). Oxaloacetate was either added directly 0.15 mM (○, △) or generated from malate (20 mM) in the presence of NAD^+ (1 mM) and pyruvate 0.4 mM and catalyzed by malate dehydrogenase (●, ▲). In addition the reaction mixture contained in 1 ml final volume the following components: 50 mM Tris–HCl buffer, pH 8; 0.75 mM $MnCl_2$, 0.75 mM ITP and tissue supernatants. Reaction was stopped by boiling and phosphoenolpyruvate determined as described under EXPERIMENTAL PROCEDURE. Each point is the average of 2 measurements.

Fig. 2. Linearity of phosphoenolpyruvate formation with respect to the amount of liver (●) and adipose tissue (○) supernatants added. Other conditions as outlined in Table I.

The equilibrium constant used for the aspartate aminotransferase system was taken from HENSON AND CLELAND[16].

RESULTS

The decarboxylation catalyzed reaction by phosphoenolpyruvate carboxylase in crude extracts

As shown in Table I, phosphoenolpyruvate formation was dependent on the presence of tissue extracts from adipose tissue or liver, on oxaloacetate, ITP, and Mn^{2+}. Replacing ITP by GTP or UTP gave lower activities and with CTP or ATP only residual ones (Table I). The concentrations of ITP and Mn^{2+} used were found to be optimal. Mn^{2+} was preferable to Mg^{2+} ions added at optimal concentration. This is in accordance with results of HOLTEN AND NORDLIE[17], for liver cytosol phosphoenolpyruvate carboxylase.

Enzymatic activities proceeded linearly for 3 and 4 min for liver and adipose tissue respectively (Fig. 1), and proportionally to the amounts of tissue extracts used for both the adipose tissue and liver enzymes (Fig. 2).

Recovery at the end of the assay of phosphoenolpyruvate added to the reaction mixture was 81%. The loss was not dependent on the presence of ITP but was a constant portion of the added phosphoenolpyruvate (Fig. 3). Since initial velocities of phosphoenolpyruvate carboxylase were measured and therefore concentrations of phosphoenolpyruvate started from zero, the loss of phosphoenolpyruvate formed during the enzymatic reaction was about 9%.

Dependence of enyzme activity upon the concentration of oxaloacetate

Initial velocity of phosphoenolpyruvate formation as a function of oxaloacetate

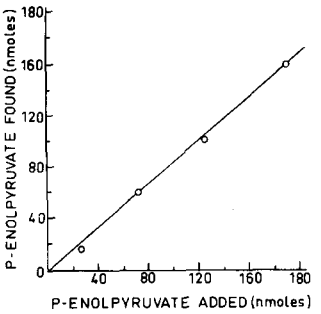


Fig. 3. Recovery of phosphoenolpyruvate added to the assay reaction mixture concentration varied from 20 to 180 nmoles, containing liver supernatant. Conditions as in Table I except that ITP was deleted.

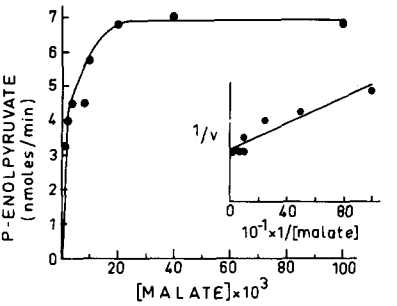


Fig. 4. Activity of adipose tissue phosphoenolpyruvate carboxylase as a function of the concentration of oxaloacetate. All other components were kept constant. Each point is the average of 2 measurements.

concentration are shown in Fig. 4. The concentrations of oxaloacetate ranged from $2.5 \cdot 10^{-5}$ to $4 \cdot 10^{-4}$ M. Above $2 \cdot 10^{-4}$ M, liver phosphoenolpyruvate carboxylase activity was markedly inhibited (Table II). On the other hand, with adipose tissue enzyme, the inhibition was smaller and inconsistent (Fig. 4, Table II), and the addition of pyruvate (0.4 mM) overcame the inhibition (Tale II). No effect of pyruvate on the inhibition of liver enzyme by oxaloacetate was evident (Table II). The K_m values obtained from the Lineweaver–Burk plots for oxaloacetate were $3.0 \cdot 10^{-4}$ M for liver enzyme and $2.6 \cdot 10^{-5}$ M for adipose tissue enzyme. The K_m value of the adipose tissue enzyme was not changed by the presence of pyruvate (data not presented).

Precursors for phosphoenolpyruvate formation

It is well known that oxaloacetate may be generated by several substrates in the

TABLE II

INHIBITION OF PHOSPHOENOLPYRUVATE FORMATION BY HIGH CONCENTRATIONS OF MALATE AND OXALOACETATE AND THE EFFECT OF PYRUVATE

Conditions and complete reaction mixture as described in Table I unless otherwise specified in the table.

Addition to reaction mixture	*Phosphoenolpyruvate formation (nmoles/min)*		
	Adipose tissue	*Liver*	
		a	*b*
None (complete)	20	24	38
Pyruvate, 0.4 μmole	19	24	
Malate, 100 μmoles	12	9	
Malate, 100 μmoles + pyruvate, 0.4 μmole	16	11	
Oxaloacetate, 0.4 μmole	13		26
Oxaloacetate, 0.4 μmole + pyruvate, 0.4 μmole	18		27

TABLE III

PRECURSORS FOR PHOSPHOENOLPYRUVATE FORMATION IN ADIPOSE TISSUE AND LIVER CYTOSOL

Adipose tissue and liver cytosol were incubated at 37° with the substrates as outlined in the table. In addition the reaction mixture contained the following components in their final concentrations: 50 mM Tris–HCl buffer, pH 8.0; 0.75 mM $MnCl_2$; 0.75 mM ITP. At the end of 7 min the reaction was stopped by boiling and phosphoenolpyruvate determined as specified under EXPERIMENTAL PROCEDURE.

Expt.	*Addition or deletion*	*Phosphoenolpyruvate formation (nmoles)*	
		Adipose tissue	*Liver*
A	Malate, 10 μmoles	71	74
	Malate, 10 μmoles + pyruvate, 0.4 μmole	103	
	Malate, 10 μmoles; ITP deleted	14	8
	Aspartate, 10 μmoles + α-ketoglutarate, 10 μmoles	56	134
	Aspartate, 10 μmoles + α-ketoglutarate, 10 μmoles; ITP deleted	15	8
B	Malate, 10 μmoles	56	
	Malate, 10 μmoles + pyruvate, 0.4 μmole	119	
	Malate, 10 μmoles; ITP deleted	8	
	Citrate, 10 μmoles	14	
	Citrate, 10 μmoles + ATP, 0.5 μmole + CoA, 0.5 μmole + $MgCl_2$, 5 μmoles	25	
	Citrate, 10 μmoles + ATP, 0.5 μmole, + CoA, 0.5 μmole + $MgCl_2$, 5 μmoles; ITP deleted	14	

cytosol. The efficacy of various sources of oxaloacetate was tested by following the formation of phosphoenolpyruvate. As shown in Table III, three systems were tested: malate, aspartate + α-ketoglutarate and citrate. In agreement with similar observations made by SHRAGO AND LARDY[18] it was found that in liver cytosol, aspartate + α-ketoglutarate supported the formation of phosphoenolpyruvate to a greater extent than malate. With adipose tissue addition of an extra amount of transaminase equalized the rate of phosphoenolpyruvate formation from α-ketoglutarate + aspartate to that obtained from malate. The results indicate that aspartate aminotransferase activity in adipose tissue cytosol is relatively low and malate may be considered the main precursor for phosphoenolpyruvate formation inside the cell.

Generation of oxaloacetate

By generating oxaloacetate from either malate or aspartate, phosphoenolpyruvate carboxykinase activity could be assayed. As shown in Table V, the ITP dependent phosphoenolpyruvate carboxylase activities measured by generating oxaloacetate were comparable to those obtained by direct addition of oxaloacetate. With malate dehydrogenase system, at optimal malate concentration the addition of pyruvate was required for maximal enzyme activity[18]. The optimal concentration of pyruvate was found to be 0.4 mM (Table IV). In the presence of pyruvate, the rate of phosphoenolpyruvate formation was linear with time for about 5 min and 8 min for liver and adipose tissue respectively (Fig. 1) and with the amount of tissue extract added.

TABLE IV

EFFECT OF PYRUVATE ON MALATE DEHYDROGENASE SYSTEM

Conditions as specified in Table I, except that oxaloacetate was replaced by malate (concentration as outlined in the table), 1 μmole NAD^+ and 2 units of malate dehydrogenase. The reaction mixture containing all the components except tissue extract and ITP was incubated at 37° for 3 min followed by the addition of the tissue extract. 1 min later phosphoenolpyruvate formation was initiated by the addition of ITP. Results are expressed as percent over control. 100% indicates between 20–40 nmoles phosphoenolpyruvate formed with adipose tissue extract and 60 to 90 nmoles of phosphoenolpyruvate formed with liver extract. Number in parentheses indicates the number of observations.

Addition or deletion	*% of control*	
	Adipose tissue	*Liver*
Malate, 10 mM	100	100
Malate, 10 mM + pyruvate, 0.2 mM	148	
Malate, 10 mM + pyruvate, 0.4 mM	195 ± 44.5 (5)	198 ± 30 (3)
Malate, 10 mM + pyruvate, 0.8 mM	170 ± 7.0 (3)	142
Malate, 20 mM	100 (3)	
Malate, 20 mM + pyruvate, 0.4 mM	177 ± 31 (4)	168 ± 38 (4)
Pyruvate, 0.4 mM; malate deleted	20	
Malate, 50 mM	130 ± 46 (3)	140
Malate, 50 mM + pyruvate, 0.4 mM	228 ± 46 (3)	227
Malate, 50 mM + pyruvate, 0,8 mM	197 ± 70 (3)	206

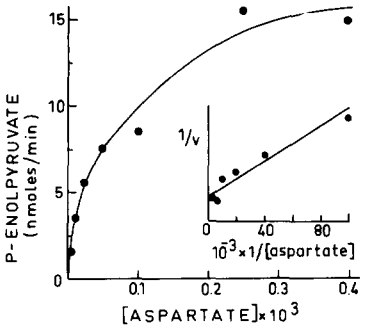
The dependence of phosphoenolpyruvate formation on malate concentration in the case of malate dehydrogenase system and of aspartate and α-ketoglutarate in the case of aspartate aminotransferase system, are shown in Figs. 5–7. With the aspartate aminotransferase system (Fig. 5) increasing aspartate and α-ketoglutarate concentration did not inhibit phosphoenolpyruvate carboxylase activity. Even at a concentration of 10^{-3} M of each of these substrates, which upon equilibrium yielded an oxaloacetate concentration of $4 \cdot 10^{-4}$ M no inhibition was apparent. This is despite the fact that a direct addition of oxaloacetate at this concentration was inhibitory for phosphoenolpyruvate carboxylase activity (Table II).

TABLE V

COMPARISON BETWEEN OXALOACETATE AND SYSTEMS WHICH GENERATE OXALOACETATE

Oxaloacetate was either added directly or replaced by (a) 20 mM malate, 1 μmole NAD^+, 2 units malate dehydrogenase with or without 0.4 mM pyruvate; (b) 1 mM aspartate, 1 mM α-ketoglutarate and 0.5 unit aspartate aminotransferase. With either of the two generating systems the complete reaction mixture (without ITP and tissue extract) was incubated for 3 min at 37°, then tissue extract was added and 1 min later phosphoenolpyruvate formation was initiated with ITP. When oxaloacetate was added, the reaction was initiated by the oxaloacetate.

Oxaloacetate added or generated	*Phosphoenolpyruvate formation (nmoles/min)*			
	Adipose tissue		*Liver*	
	With ITP	*Without ITP*	*With ITP*	*Without ITP*
Oxaloacetate	10	1.2	41.5	2.5
Generated from malate	8.5	1.7	25.5	2.5
Generated from malate + pyruvate	11.2	2.2	43	5
Generated from aspartate			41	2



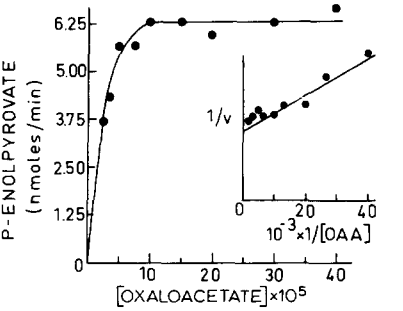


Fig. 5. Activity of liver phosphoenolpyruvate carboxylase as a function fo the concentrations of α-ketoglutarate and aspartate. The concentrations of these 2 substrates were kept equimolar. Other conditions pertaining to this system are outlined in Table V.

Fig. 6. Activity of adipose tissue phosphoenolpyruvate carboxylase as a function of malate concentration used to generate oxaloacetate. Otherwise conditions are as outlined in Table V, except that no pyruvate was added. Each point is the average of 2 measurements.

The K_m values for oxaloacetate, calculated from the equilibrium constant of aspartate aminotransferase were $1.2 \cdot 10^{-5}$ M and $2.7 \cdot 10^{-5}$ M (Table VI) for liver and adipose tissue respectively; these values were close to those found for adipose tissue enzyme with oxaloacetate added directly, but differed markedly from those found for liver with the direct addition of oxaloacetate. This low K_m value also agrees with the findings recently reported for a purified enzyme preparation from rat liver cytosol[25], and differs by an order of magnitude from the value reported by CHANG *et al.*[9]. The present K_m value for oxaloacetate approaches the physiological concentration of this substrate[22–24].

TABLE VI

K_m VALUES OF PHOSPHOENOLPYRUVATE CARBOXYLASE FOR OXALOACETATE AND MALATE

Complete reaction mixtures as outlined in Table V. Oxaloacetate concentration was ranged from $1.25 \cdot 10^{-5}$ to $4 \cdot 10^{-4}$ M. With malate dehydrogenase system the concentration of malate was varied from $5 \cdot 10^{-4}$ to 10^{-1} M. With malate dehydrogenase system and pyruvate (0.4 mM) the concentration of malate was varied from 10^{-4} to $4 \cdot 10^{-1}$ M. With aspartate aminotransferase system aspartate and α-ketoglutarate concentrations ranged from $5 \cdot 10^{-6}$ to 10^{-3} M, and both substrates were kept at equimolar concentrations. With oxaloacetate generating systems the K_m values for oxaloacetate were derived from the known equilibrium constants of these systems[15,16].

Oxaloacetate added or generated	*K_m value (mM)*			
	For oxaloacetate		*For malate*	
	Adipose tissue	*Liver*	*Adipose tissue*	*Liver*
Oxaloacetate	$2.6 \cdot 10^{-5}$	$3 \cdot 10^{-4}$		
Generated from malate	$1.25 \cdot 10^{-5}$	$1.5 \cdot 10^{-5}$	$1.4 \cdot 10^{-3}$	$1.9 \cdot 10^{-3}$
Generated from malate + pyruvate			$2.3 \cdot 10^{-4}$	$4.5 \cdot 10^{-4}$
Generated from aspartate	$2.7 \cdot 10^{-5}$	$1.4 \cdot 10^{-5}$		

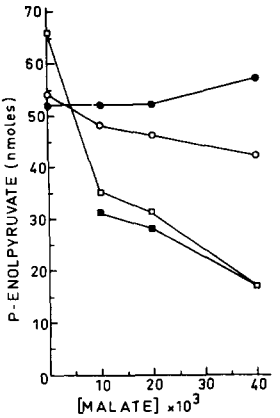


Fig. 7. Effect of malate and pyruvate on phosphoenolpyruvate formation from oxaloacetate catalyzed by liver (□, ■) and adipose tissue (○, ●) supernatants. Components of reaction mixture as outlined in Table I. Increasing concentrations of malate were added to the assay mixture as described in the figure, without (open symbols) and with (closed symbols) 0.4 mM pyruvate.

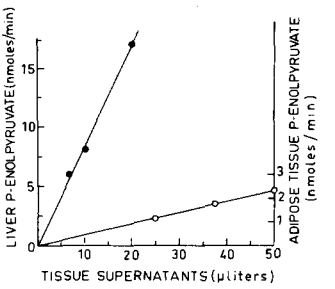


Fig. 8. Activity of phosphoenolpyruvate carboxylase in liver (●) and adipose tissue (○) with respect to the amount of enzyme tissue extracts is measured by generating oxaloacetate from low concentration of malate (0.25 mM). In addition the reaction mixture contained in 1 ml the following components: 50 mM Tris–HCl buffer, pH 8; 1 mM NAD^+, 0.75 mM $MnCl_2$, 0.75 mM ITP, 0.4 mM pyruvate, 1 unit malate dehydrogenase and 1 unit lactate dehydrogenase. Other details as described under EXPERIMENTAL PROCEDURE.

With the malate dehydrogenase system an inhibition of phosphoenolpyruvate carboxylase became apparent upon increasing the malate concentrations over $4 \cdot 10^{-2}$ M (Fig. 6). The inhibition by malate was not due to a generation of higher concentration of oxaloacetate but to an inhibitory effect of malate, since the addition of malate to a reaction mixture containing oxaloacetate, resulted in a marked decrease of phosphoenolpyruvate formation (Table II). Liver phosphoenolpyruvate carboxylase is inhibited even by lower concentrations of malate. 10 mM malate reduced the activity to 50% (Fig. 7) and the addition of pyruvate did not affect this inhibition. This concentration of malate had little effect on the adipose tissue enzyme (Fig. 7.) Considerable inhibition of the adipose tissue enzyme could only be shown with 100 mM malate (Table II), and the addition of pyruvate (0.4 mM) overcame the inhibition (Fig. 7, Table II).

Despite the inhibition of liver phosphoenolpyruvate carboxylase by malate the K_m value for oxaloacetate, calculated from the equilibrium constant of malate dehydrogenase, is low (Table VI), similar both to the value obtained by the aspartate aminotransferase system for the liver enzyme and to the value obtained for adipose tissue enzyme with all 3 systems (oxaloacetate added directly, aspartate aminotransferase system and malate dehydrogenase system). Half maximal activities of adipose tissue and liver phosphoenolpyruvate carboxylase were measured with 0.23 mM and 0.45 mM malate respectively, provided that pyruvate (0.4 mM) was present (Table VI). At this low malate concentration (0.25 mM) the activity of phosphoenolpyruvate carboxylase was linear with respect to the amount of tissue extract added. This was true for both liver and adipose tissue enzymes (Fig. 4B).

TABLE VII

COMPARISON BETWEEN CARBOXYLATION AND DECARBOXYLATION CATALYZED REACTIONS OF PHOSPHOENOLPYRUVATE CARBOXYLASE

Adipose tissue extracts prepared from animals treated as outlined in the table were assayed for phosphoenolpyruvate carboxylase activity. A simultaneous assay of the carboxylation reaction and decarboxylation reaction were carried out with each extract separately. For the measurement of the decarboxylation catalyzed reaction oxaloacetate was generated from malate (20 mM) in the presence of pyruvate 0.4 mM. The carboxylation reaction was performed as described under EXPERIMENTAL PROCEDURE. One unit of enzyme measured by the carboxylation reaction is the amount which catalyzed the fixation of 1 μmole $H^{14}CO_3^-$ in the presence of phosphoenolpyruvate, Mn^{2+} and IDP at 37°. One unit of enzyme measured by the decarboxylation reaction is the amount of tissue extract which catalyzed the ITP dependent formation of 1 μmole of phosphoenolpyruvate per min at 37°. Results are the means ± S.E.of the number of animals indicated in parentheses.

Treatment of animals	*Phosphoenolpyruvate carboxylase activity*			
	Adipose tissue (munits/g tissue)		*Liver (units/g tissue)*	
	Carboxylation	*Decarboxylation*	*Carboxylation*	*Decarboxylation*
Intact fed (3)	39 ± 2.04	84 ± 16.6	2.33 ± 0.067	3.84 ± 0.26
Intact 24 h fasted (5)	288 ± 33	464 ± 58	7.76 ± 0.71	9.18 ± 0.9
Adrenalectomized fed (5)	277 ± 26	468 ± 95		
Adrenalectomized 24 h fasted (5)	486 ± 38	812 ± 47	7.98 ± 0.49	11.1 ± 0.57

Ratio of decarboxylation to carboxylation reactions catalyzed by phosphoenolpyruvate carboxylase

As pointed out earlier, the main purpose of the present study was to compare the rate of the reactions catalyzed by phosphoenolpyruvate carboxylase in the two directions with crude liver and adipose tissue extracts. With liver phosphoenolpyruvate carboxylase this comparison was not feasible as measurements of maximal activities of the decarboxylation catalyzed reaction could not be ensured satisfactorily.

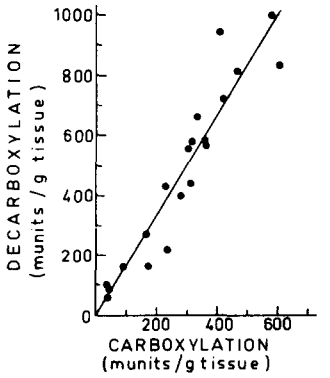


Fig. 9. Relationship of carboxylation to decarboxylation catalyzed reactions. Adipose tissue supernatants were obtained from animals treated variously as described in Table VII. Phosphoenolpyruvate carboxylase activity was assayed simultaneously in both directions. The slope of the regression line and the intercept were calculated according to the following equations[30]. $\Sigma y = aN + b\Sigma x$ and $\Sigma xy = a\,x + b\Sigma x^2$.

Nevertheless it is worth noting that the activities obtained (Table VII) were very close or slightly higher than the activities reported by FOSTER *et al.*[19]. With adipose tissue on the other hand maximal activities measured simultaneously in both directions were made, and presented in Table VII. For comparison these measurements were made in adipose tissue extracts obtained from groups of animals that underwent various treatments. As shown in Table VII changes in enzyme activity induced by various treatments *in vivo* were similar, whether measured in the backward or forward directions.

The individual figures of activities measured in the direction of decarboxylation were plotted against the simultaneous measured activities by the carboxylation method (Fig. 9). The regression line obtained indicates clearly a linear relationship between the two assays over a wide range of activities assayed. Each of the reactions was measured at near its own optimal pH. In the case of the decarboxylation catalyzed reaction the pH optimum was found to be around 8 for all three systems used (oxaloacetate added directly, malate dehydrogenase system and aspartate aminotransferase system) while the carboxylation reaction was measured at pH 7.0. Under these conditions the slope of the regression line indicates a ratio of 1.66 between the decarboxylation to carboxylation catalyzed reactions. This ratio is far below the ratio reported for the purified phosphoenolpyruvate carboxylase from pig liver mitochondria[9].

DISCUSSION

In their recent review on gluconeogenesis SCRUTTON AND UTTER[20] pointed out that the maximal capacity of a tissue to carry out a given sequence of reactions is dependent on the amounts of the key enzymes participating in it. In that respect, alteration in the amounts of the key enzymes would contribute to alterations in the overall capacity and thus to the regulation of the pathway. Phosphoenolpyruvate carboxylase is considered as a key enzyme in gluconeogenesis in the liver and in glyceroneogenesis in adipose tissue[2,20,21]. Changes in enzyme activities, brought about by various *in vivo* treatments correlate closely with changes in the overall activity of the pathway[8,20]. However, its amount, as measured by the maximal activity, exceeds the overall capacity of the liver to produce glucose by a factor of 2 (ref. 20), and the rate of glyceroneogenesis in adipose tissue by a factor of 7 (Table VIII, ref. 8). This implies either that phosphoenolpyruvate carboxylase is a key enzyme but that under all circumstances *in vivo* the enzyme functions at a fraction of its maximal activity. Alternatively, that phosphoenolpyruvate carboxylase catalyzed the formation of phosphoenolpyruvate at its maximal activity *in vivo*, but that it is not the key site of regulation of the gluconeogenic pathway. Analysis of the present experimental data support the view that the activity of phosphoenolpyruvate carboxylase inside the cell never exceeds one half of its measured maximal activity.

In adipose tissue, phosphoenolpyruvate carboxylase is located in the cytosol[2] while pyruvate, a glyceroneogenic substrate[2,6-8] is carboxylated in the mitochondria[26,27]. Recently it was shown [28]that if adipose tissue mitochondria were incubated with $^{14}CO_2$ and pyruvate, the label appeared in citrate and malate which subsequently diffused out to the suspending medium. Consequently, by diffusing out of the mitochondria these two substrates could generate oxaloacetate *in vivo*. Since, as shown in

Table III only malate but not citrate supported phosphoenolpyruvate formation and it was also preferable to aspartate, malate may be considered as the main precursor for phosphoenolpyruvate formation in adipose tissue.

The existence of an excess amount of dehydrogenases in animal cells enables to maintain these systems at a near equilibrium state *in vivo*[31]. In that respect, our malate dehydrogenase system supplemented with pyruvate and brought to an equilibrium resembles the *in vivo* system, phosphoenolpyruvate carboxylase activity becomes therefore dependent on the concentration of malate. Thus, 1/2–1/3 of the enzyme maximal activity was measured at a physiological concentration of malate[22–24,29].

In adipose tissue malate concentration does not change by various *in vivo* treatments[29], while slight changes were reported in liver[22–24]. Therefore the activity of phosphoenolpyruvate carboxylase *in vivo* must depend principally on the ratio of NAD^+ to NADH in the cytosol. Yet as shown in Table VI, even under optimal conditions of this ratio, as exemplified by using an optimal concentration of reducible substrate (pyruvate) the activity of phosphoenolpyruvate carboxylase was only 1/2–1/3 of its maximal activity. Consequently it is clearly evident that under *in vivo* conditions the enzyme never functions at a rate which exceeds one half of its maximal activity.

Despite the fact that only a fraction of enzyme activity is measured at physiological concentration of malate the linear relationship between enzyme amounts and activities (Fig. 8) indicates the significance that changes in enzyme amount may bear on the regulation of phosphoenolpyruvate formation under conditions which resemble the *in vivo* situation.

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