Control of Phosphoenolpyruvate Synthesis in Guinea-Pig Mitochondria

By M. B. WILSON*

Department of Biochemistry, University of Oxford, South Parks Road,
Oxford OX2 6HE, U.K.

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1. The synthesis of phosphoenolpyruvate and the O_2 consumption from the tricarboxylic acid-cycle intermediates citrate, α -oxoglutarate, malate and succinate by guinea-pig mitochondria were compared. Malate was the most effective of these precursors; there was no synthesis of phosphoenolpyruvate from succinate. 2. The addition of palmitate, acetoacetate and ATP enhanced the synthesis of phosphoenolpyruvate from citrate and α -oxoglutarate. Palmitate and ATP increased the O_2 consumption, whereas acetoacetate had no effect on this parameter. 3. Octanoate depressed the synthesis of phosphoenolpyruvate from citrate, α -oxoglutarate and malate and increased the O_2 consumption. Pentenoic acid had no effect on phosphoenolpyruvate synthesis from any of the substrates used, although it increased the uptake of O_2 . These findings might be relevant to the control of gluconeogenesis *in vivo*.

The metabolic pathway for the synthesis of glucose from glucogenic amino acids is well established. The enzymes involved in this pathway are distributed between the mitochondria and the cytoplasm, the first set of reactions taking place in the mitochondria. The limit of mitochondrial synthesis is dependent on the distribution of the key glucogenic enzyme phosphoenolpyruvate carboxylase [GTP-oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32]. Thus in some species, e.g. the rat, where this enzyme is in the cytoplasm (Nordlie & Lardy, 1963), the mitochondrial reactions generate malate and aspartate, which diffuse across the mitochondrial membrane and are converted into oxaloacetate by cytoplasmic malate dehydrogenase (EC 1.1.1.37) and transaminase (EC 2.6.1.1) respectively. The oxaloacetate is then converted into phosphoenolpyruvate via the enzyme carboxylase and eventually into glucose (Lardy *et al.*, 1965; Walter *et al.*, 1966). In those species where the enzyme is mainly mitochondrial, e.g. rabbit and guinea pig (Nordlie & Lardy, 1963), phosphoenolpyruvate is formed in the mitochondria and must be transported into the cytoplasm where it is converted into glucose. It is generally agreed that the synthesis of phosphoenolpyruvate is one of the first important control points in the synthesis of glucose from amino acids. Hence in those species in which it is synthesized in the mitochondria, these isolated particles provide a simplified system to study factors that affect the formation of this key intermediate. It is known that elevation of free fatty acid concentrations stimulates glucose production *in vivo* (Friedmann *et al.*, 1967), and there is rapid mobilization of fatty

acids in conditions in which gluconeogenesis is enhanced (Long *et al.*, 1940; Renold *et al.*, 1953; Krebs, 1964). Long-chain fatty acids have also been reported to stimulate gluconeogenesis from lactate in the perfused liver (Struck *et al.*, 1965; Williamson *et al.*, 1968).

The present study was undertaken to examine the effects of fatty acids and the products of fatty acid oxidation on phosphoenolpyruvate synthesis. Guinea-pig mitochondria were used as they have a high concentration of phosphoenolpyruvate carboxylase.

Materials and Methods

Reagents and enzymes were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; pentenoic acid was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Mitochondria were prepared from liver of fed male guinea pigs by the technique of Schneider & Hogeboom (1950) in buffer consisting of 0.25 M-sucrose – 0.01 M-Tris – HCl (pH 7.4) – 0.001 M-EDTA. They were washed once in the same buffer and suspended in 2–2.5 ml of 0.15 M-KCl/g of tissue homogenized. The composition of the medium was essentially that described by Stanbury & Mudge (1954) (see Table 1); 2 ml of mitochondrial suspension was used per flask. The standard Warburg technique was used to measure the O_2 consumption (Umbreit, 1964). Incubation was for 30 min at 30°C, after which the vessels were removed on to ice and the reaction was stopped by the addition of 1 ml of 30% (w/v) $HClO_4$. The protein precipitate was removed by centrifugation for 10–15 min at 1000g and 2 ml portions of supernatant were neutralized with 20% (w/v) KOH.

* Present address: Department of Biochemistry, University of the West Indies, Mona, Kingston 7, Jamaica, West Indies.

The supernatant obtained after removal of the KClO_4 was used for the various determinations.

All substrates were assayed enzymically. Phosphoenolpyruvate was determined by the method of Czok & Eckert (1963), acetoacetate and 3-hydroxybutyrate by the method of Williamson *et al.* (1962) and malate by the method of Hohorst *et al.* (1959).

Results

Effect of acetoacetate on phosphoenolpyruvate synthesis from tricarboxylic acid-cycle intermediates

Table 1 shows the synthesis of phosphoenolpyruvate in guinea-pig mitochondria from four tricarboxylic acid-cycle intermediates and the effect of addition of acetoacetate on this synthesis. Under these conditions, malate produced two to three times as much phosphoenolpyruvate as did α -oxoglutarate or citrate, despite the fact that the rates of O_2 uptake are equal. Succinate was oxidized rapidly as indicated by the high rate of O_2 consumption without an increase in phosphoenolpyruvate synthesis. The addition of acetoacetate to citrate and α -oxoglutarate produced a 100% increase in phosphoenolpyruvate synthesis without significantly affecting the O_2 consumption. No stimulation of either phosphoenolpyruvate synthesis or O_2 consumption was obtained when malate or succinate was the substrate. The amount of 3-hydroxybutyrate formed from acetoacetate was greater in the presence of citrate than of α -oxoglutarate or malate. There was almost quantitative (70%) conversion of succinate into malate both in the presence and in the absence of acetoacetate.

Effect of fatty acids on phosphoenolpyruvate synthesis from tricarboxylic acid-cycle intermediates

Table 2 compares the effect of palmitate and octanoate (final concns. 0.75 mM and 1 mM respectively) on the synthesis of phosphoenolpyruvate. This concentration of palmitate resulted in a threefold increase in phosphoenolpyruvate synthesis from citrate, a fivefold increase from α -oxoglutarate (cf. Table 1) and also increased the O_2 uptake. When malate was the substrate, there was a decrease in both the O_2 consumption and phosphoenolpyruvate synthesis. The addition of octanoate, however, decreased the synthesis of phosphoenolpyruvate from citrate, α -oxoglutarate and malate (by 50–66%) but increased the O_2 consumption fourfold for citrate and threefold for α -oxoglutarate and malate. Neither palmitate nor octanoate had any effect on the synthesis of phosphoenolpyruvate when succinate was the substrate. The total amount of ketone bodies produced in the presence of palmitate was not significantly different from the endogenous value, whereas the yield from

Table 1. *Effect of acetoacetate on phosphoenolpyruvate synthesis and respiration from tricarboxylic acid-cycle intermediates*

Substrate	Addition (final concn. 2.9 mM)	Phosphoenolpyruvate synthesis	O_2 uptake	Hydroxybutyrate ($\mu\text{mol}/\text{flask}$)	Malate ($\mu\text{mol}/\text{flask}$)
Citrate	—	10.6 ± 1.2 (6)	18.0 ± 1.6 (6)	—	2.0 (3)
	Acetoacetate	17.9 ± 3.3 (4)	21.0 ± 1.9 (4)	3.4 (3)	2.2 (3)
α -Oxoglutarate	—	13.2 ± 1.6 (7)	21.8 ± 2.1 (7)	—	2.8 (3)
	Acetoacetate	22.2 ± 3.2 (4)	23.2 ± 1.8 (4)	2.6 (3)	3.5 (3)
Malate	—	28.2 ± 3.3 (7)	19.9 ± 2.6 (7)	—	2.8 (3)
	Acetoacetate	31.7 ± 5.2 (4)	21.8 ± 3.3 (4)	1.9 (3)	—
Succinate	—	5.0 ± 0.9 (5)	46.6 ± 3.8 (5)	—	22.1 (3)
	Acetoacetate	5.4 ± 0.9 (4)	45.2 ± 1.6 (4)	1.4 (3)	21.5 (3)

The reaction mixture contained: Tris-HCl buffer, pH 7.4 (60 μmol); KH_2PO_4 - K_2HPO_4 (20 μmol); MgCl_2 (5 μmol); 60–80 mg dry wt. of guinea-pig liver mitochondria; substrate (30 μmol); as the potassium salt in a final volume of 3.4 ml. Incubation was for 30 min at 30°C. Results are expressed as $\mu\text{mol}/\text{g}$ dry wt. of mitochondria $\pm$ S.E.M., with the numbers of experiments in parentheses.

Table 2. *Effect of palmitate and octanoate on phosphoenolpyruvate synthesis and respiration from tricarboxylic acid-cycle intermediates*

Conditions and units were the same as in Table 1; the final concentrations of palmitate and octanoate were 0.75 mM and 1.0 mM respectively. The total amount of ketone bodies produced in the absence of substrate was 0.27 μ mol.

Substrate	Addition	Phosphoenolpyruvate synthesis	O ₂ uptake	3-Hydroxybutyrate (μ mol/flask)	Acetoacetate (μ mol/flask)	Total ketone bodies (μ mol/flask)
Citrate	Palmitate	32.3 $\pm$ 2.4 (3)	55.3 $\pm$ 10.6 (3)	0.1 (3)	0.28 (3)	0.38
	Octanoate	5.1 $\pm$ 1.0 (4)	71.1 $\pm$ 12.3 (4)	3.7 (3)	2.0 (3)	5.7
α -Oxoglutarate	Palmitate	68.8 $\pm$ 10.1 (5)	99.4 $\pm$ 16.0 (5)	0.07 (3)	0.29 (3)	0.36
	Octanoate	4.6 $\pm$ 0.6 (4)	61.2 $\pm$ 6.9 (4)	1.7 (3)	2.6 (3)	4.3
Malate	Palmitate	13.1 $\pm$ 2.0 (4)	12.9 $\pm$ 3.0 (4)	0.06 (3)	0.21 (3)	0.27
	Octanoate	9.6 $\pm$ 2.1 (4)	65.4 $\pm$ 5.9 (4)	1.2 (3)	0.88 (3)	2.1
Succinate	Palmitate	4.3 $\pm$ 0.6 (3)	67.8 $\pm$ 15.7 (3)	0.1 (3)	0.07 (3)	0.17
	Octanoate	3.2 $\pm$ 0.4 (3)	45.9 $\pm$ 1.8 (3)	0.24 (3)	0.73 (3)	0.97

octanoate when citrate or α -oxoglutarate was the substrate represents almost complete oxidation of this fatty acid to ketone bodies (96 and 70% respectively). Malate was less effective in causing the complete oxidation of octanoate to acetoacetate and 3-hydroxybutyrate.

Effect of pentenoic acid and ATP on phosphoenolpyruvate synthesis from tricarboxylic acid-cycle intermediates

Table 3 shows that pentenoic acid, an inhibitor of fatty acid oxidation in rat liver mitochondria (Senior *et al.*, 1968; Corredor *et al.*, 1969; Wilson, 1973), had no effect on the synthesis of phosphoenolpyruvate from any of the substrates used, although it stimulated the O₂ consumption. The addition of ATP (1.5 mM) stimulated the synthesis of phosphoenolpyruvate from citrate, α -oxoglutarate and malate, and also increased the O₂ consumption.

Discussion

The results show that the tricarboxylic acid-cycle intermediates citrate, α -oxoglutarate and malate are good precursors of phosphoenolpyruvate synthesis in guinea-pig mitochondria. Of these substrates malate generated the highest concentrations of phosphoenolpyruvate. This suggests that adequate GTP was produced from the oxidation of malate through formation of ATP and the nucleoside diphosphokinase. This enzyme is present in high concentrations in guinea-pig mitochondria (Garber & Hanson, 1971). The fact that exogenous ATP stimulates phosphoenolpyruvate synthesis (Table 3) confirms the importance of this enzyme in the production of nucleotides for the synthesis of phosphoenolpyruvate. Most of the succinate was recovered as malate, suggesting that further oxidation was inhibited at this point. This inhibition is probably due to the fact that succinate inhibits the re-oxidation of NADH and thus decreases the concentration of oxaloacetate (Krebs *et al.*, 1961; Gevers, 1967).

The O₂ consumption and the amount of phosphoenolpyruvate formed from malate are low compared with that reported by Bartley & Dean (1969) for rat liver mitochondria, where none of the enzyme carbonylase is supposed to occur. This may be due to the higher concentrations of mitochondria used in these experiments, since both the respiration (Hedekov & Esmann, 1966) and protein-synthetic capacity of cells (Bhargava & Bhargava, 1962) have been shown to decrease with increasing cell concentrations *in vitro*. The observed decrease in synthesis from malate when mitochondria are uncoupled with palmitate differs from that reported for dinitrophenol-uncoupled mitochondria, in which there is increased synthesis (Davis & Gibson, 1969). These authors suggest that

Table 3. *Effect of pentenoic acid and ATP on phosphoenolpyruvate synthesis from tricarboxylic acid-cycle intermediates*

Conditions and units were the same as in Table 1. The final concentrations of pentenoic acid and ATP were 1.1 mM and 1.5 mM respectively.

Substrate	Addition	Phosphoenolpyruvate synthesis	O ₂ uptake
Citrate	Pentenoic acid	10.5 ± 1.7 (5)	39.6 ± 6.8 (5)
	ATP	27.5 ± 5.3 (3)	44.0 ± 5.0 (3)
α -Oxoglutarate	Pentenoic acid	15.2 ± 1.9 (3)	50.5 ± 7.6 (3)
	ATP	43.5 ± 9.5 (3)	43.3 ± 3.2 (3)
Malate	Pentenoic acid	21.1 ± 2.2 (3)	25.5 ± 2.3 (3)
	ATP	48.3 ± 1.3 (3)	31.4 ± 3.4 (3)
Succinate	Pentenoic acid	2.8 ± 0.2 (3)	74.4 ± 1.0 (3)

formation of phosphoenolpyruvate from this substrate is dependent on ATP generated by decarboxylation of oxaloacetate to pyruvate and oxidation in the tricarboxylic acid-cycle. Palmitate can be activated in mitochondria uncoupled with dinitrophenol via the GTP thiokinase (Lehninger, 1945; Rossi & Gibson, 1964). Thus it is likely that in fatty acid-uncoupled mitochondria the palmitoyl-CoA formed blocks formation of ATP by inhibiting citrate synthase (Wieland & Weiss, 1963; Tubbs, 1963).

The effects of acetoacetate and fatty acids on phosphoenolpyruvate synthesis from citrate and α -oxoglutarate are probably explicable by the effect these substrates might have in altering the $[NAD^+]/[NADH]$ ratio in mitochondria (Williamson *et al.*, 1967). This ratio controls the steady state of oxaloacetate and hence phosphoenolpyruvate formation. The addition of acetoacetate would tend to increase this ratio by the equilibrium reaction across the enzyme β -hydroxybutyrate dehydrogenase. This would result in increased concentrations of oxaloacetate and phosphoenolpyruvate synthesis. The fact that citrate and α -oxoglutarate produced more 3-hydroxybutyrate than either malate or succinate where there is no stimulation of synthesis supports this suggestion.

Oxidation of octanoate would tend to decrease this ratio and decrease the synthesis of phosphoenolpyruvate. This decrease is most evident when α -oxoglutarate, malate and citrate were the substrates. Acetylcarnitine and butyrylcarnitine (final concn. 5 mM) produced the same inhibitory effect on phosphoenolpyruvate synthesis (M. B. Wilson, unpublished work). The final ratio of [hydroxybutyrate]/[acetoacetate] is probably representative of both the amount of octanoate oxidized to ketone bodies and the NADH generated by oxidation of the substrate. This could explain the differences in this ratio in the presence of α -oxoglutarate and of citrate where there is complete oxidation of the octanoate to ketone

bodies. The lack of the effect of pentenoate may be because it can both increase the $[NAD^+]/[NADH]$ ratio by inhibiting endogenous fatty acid oxidation and decrease it by its oxidation in mitochondria (Corredor *et al.*, 1969).

In the presence of palmitate the O₂ consumption from citrate and α -oxoglutarate was increased without increasing significantly the total amount of ketone bodies above endogenous values, confirming that oxidative phosphorylation was uncoupled (Lehninger & Remmert, 1959). The accelerated electron transfer would increase the $[NAD^+]/[NADH]$ ratio, GTP formation linked to the oxidation of α -oxoglutarate (Sanadi *et al.*, 1956), and would result in increased phosphoenolpyruvate synthesis.

The different effects shown by almost equal concentrations of octanoate and palmitate may be explained by the fact that higher concentrations of short-chain fatty acids than of long-chain fatty acids are required to uncouple oxidative phosphorylation and stimulate adenosine triphosphatase activity (Pressman & Lardy, 1956; Borst *et al.*, 1962). Again, in mitochondria uncoupled with fatty acids, fatty acid activation via the ATP thiokinase is blocked (Cross *et al.*, 1949; Kennedy & Lehninger, 1951). Fatty acids may, however, be activated by the GTP thiokinase. This enzyme is insensitive to uncoupling agents (Van Den Berg, 1965), and competition for GTP between thiokinase and carboxylase could control the amount of acetyl-CoA generated and also divert oxaloacetate to phosphoenolpyruvate formation.

The present work demonstrates a relationship between fatty acid metabolism and the formation of an important intermediate in gluconeogenesis. These results may be relevant to the understanding of the process *in vivo*.

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