Regulation of Phosphoenolpyruvate Synthesis from Succinate in Guinea Pig Liver Mitochondria[1] (36341)

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The synthesis of phosphoenolpyruvate (PEP) from oxaloacetate (OAA) is catalyzed by phosphoenolpyruvate carboxykinase [GTP:oxaloacetate carboxy-lyase (transphosphorylating EC 4.1.1.32)] and is one of the key reactions in the pathway of gluconeogenesis. This enzyme has been shown to be present exclusively in the mitochondria of chicken and rabbit liver while it is found almost exclusively in the cytosol of rat liver (1–3). In guinea pig liver the phosphoenolpyruvate carboxykinase is present both in the mitochondria and in the cytosol (2). The phosphoenolpyruvate carboxykinase (PEPcK) in the cytosol is readily responsive to metabolic, hormonal, and dietary modulation, while the activity of the mitochrondrial enzyme remains essentially unchanged (4, 5). Mitochondrial PEP synthesis requires GTP which can be generated by substrate level phosphorylation from α-ketoglutarate (6–8) or by nucleosidediphosphate kinase (EC 2.7.4.6) from ATP (9–11).

Major studies with guinea pig liver mitochondria concerning PEP synthesis from a variety of substrates have been previously reported (1, 2, 8, 12–16). The regulation and synthesis of PEP from succinate in the absence of substrate level phosphorylation which generates GTP has not yet been well studied. This investigation therefore deals with the above-mentioned regulation through control of the substrate equilibrium by malonate, 2,4-dinitrophenol (DNP), fatty acids, ATP, ADP, and rotenone.

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Materials and Methods. Male albino guinea pigs (weighing 275–450 g) were used in all experiments. The isolation of intact liver mitochondria; incubation; processing of samples; and analysis for $^{14}CO_2$-trapped, malate, citrate, acetoacetate, β-hydroxybutyrate, phosphoenolpyruvate, and mitochondrial nitrogen were performed by methods previously described (17–19).

With the exception of the polarographic measurements shown in Table III all the incubation mixtures contained 6.7 m*M* succinate, 10.0 m*M* $MgSO_4$, 13.3 m*M* potassium phosphate, and 13.3 m*M* triethanolamine buffers at pH 7.3. Any further additions are given as footnotes to the tables. Each incubation contained mitochondria isolated from 0.5 g of liver and suspended in 0.5 ml of 0.25 *M* sucrose. Rates are expressed as micromoles per milligram nitrogen found in 0.5 ml of final mitochondrial suspension per incubation time. Final incubation volumes were adjusted to 3.0 ml with 0.15 *M* KCl as previously reported (17). Tables I, II, V, VI, and VII show data from typical experiments run in duplicate.

(1-^{14}C) Succinate and (1,4-^{14}C) succinate were obtained from Tracer Lab. (Waltham, MA) (^{14}C) $NaHCO_3$ was obtained from Volk Radiochemical Co. (Skokie, IL) and was diluted with unlabeled $KHCO_3$ to give a final concentration of 0.2 to 0.6 μCi/μmole. Acylcarnitines were a generous gift of Dr. Yuzo Kawashima of Otsuka Pharmaceutical Factory, Naruto, Japan. All other reagents were of the highest purity commercially obtainable.

Oxygen consumption and P/O ratios were measured on a Gilson Medical Electronics

TABLE I. Effect of ADP and ATP on Synthesis of Phosphoenolpyruvate from Succinate in Guinea Pig Liver Mitochondria.

Addition to system[a] (m*M*)	Metabolite content (μmoles/3 mg of N/30 min); found: PEP	Malate
None	0.18	12.0
ADP, 3.3	3.63	8.1
ATP, 3.3	2.07	9.6

[a] Reaction mixture contained 13.3 m*M* $KHCO_3$ and 3.0 mg of mitochondrial nitrogen.

Oxygraph Model KM.

Results. Effect of ATP and malonate on succinate metabolism and PEP synthesis in guinea pig liver mitochondria. The synthesis of PEP from succinate in the presence of ATP or ADP is shown in Table I. In the absence of adenine nucleotides, PEP formation from succinate is very low; this can be attributed to the low concentration of guanidine nucleotides in mitochondria (6, 7). In the presence of 3.3 m*M* ATP or 3.3 m*M* ADP there was an 11- and 20-fold increase in PEP formation, respectively. This stimulation of PEP synthesis is attributed to GTP formation by nucleosidediphosphate kinase from intramitochondrial ATP. The larger increase in PEP formation in the presence of ADP may be due to faster removal of reduced pyridine nucleotides from the electron transport chain during phosphorylation of ADP to ATP. This decreased NADH/NAD^+ ratio favors oxaloacetate formation from malate. The decrease in malate levels in the presence of ADP or ATP is due to increased formation of PEP.

Malonate is known to be a competitive inhibitor of succinate dehydrogenase [succinate:FAD oxdioreductase (EC 1.3.99.1)] and if the rate of succinate oxidation were limiting for PEP synthesis, malonate should decrease PEP levels.

The results in Table II show that in the presence of 13.3 m*M* malonate there was a 2.8-fold increase in the PEP formation from succinate. This stimulation of PEP formation by malonate is attributed to incomplete inhibition of succinate dehydrogenase with continued oxidation of succinate at a low rate. This slow leak of succinate is accompanied by oxidation of the cytochrome chain and NAD^+ synthesis which favors the conversion of malate to oxaloacetate. Although less malate is formed, a greater fraction of it appears as PEP. Results in Table III show that 20 m*M* malonate greatly inhibited respiration of succinate both in state 3 and in the presence of an uncoupler (DNP). The controlled respiration, state 4, remained unchanged however, even in the presence of malonate. This demonstrates a slow leak of substrate for oxidation.

Effect of 2,4-dinitrophenol (DNP) on PEP synthesis from succinate. The stimulation of PEP synthesis by DNP in liver mitochrondria from rabbit (20, 21) and guinea pig has been previously reported (22). Addition of 0.033 m*M* DNP greatly stimulated PEP formation from succinate (Table IV). The decrease in malate content in the presence of DNP can be accounted for in part by increased conversion to PEP. A decrease in the ratio of β-hydroxybutyrate to acetoacetate was observed in the presence of DNP. The decrease in the β-hydroxybutyrate/acetoacetate ratios indicate that the concentration ratios of NADH/NAD^+ are decreased (23) and this change in the oxidation–reduction state of pyridine nucleotides leads to increased synthesis of PEP.

Effect of fatty acids and rotenone on PEP synthesis from succinate. PEP synthesis is

TABLE II. Effect of Malonate on (1-^{14}C) Succinate Oxidation and Phosphoenolpyruvate Synthesis.

Addition to system[a]	Metabolite content (μmoles/2.1 mg of N/18 min[b]) Found: PEP	Malate	$^{14}CO_2$ trapped
None	0.57	9.4	1.13
Malonate, 13.3 m*M*	1.64	0.21	2.94

[a] Reaction mixture contained 3.3 m*M* ATP, 6.7 m*M* (1-^{14}C) succinate (sp act 37,890 dpm/μmole) and 2.1 mg of mitochondrial nitrogen.

[b] Incubation time, 18 min.

TABLE III. Respiration and Oxidative Phosphorylation of Succinate by Guinea Pig Liver Mitochondria in the Presence of Malonate.

Parameters examined[b]	Experimental[a, c] None (6)	With malonate, 20 mM (6)
State 3 $\dot{Q}_{O_2}$	43.2 ± 2.7	27.5 ± 1.8
State 4 $\dot{Q}_{O_2}$	12.7 ± 0.7	12.7 ± 0.6
DNP $\dot{Q}_{O_2}$	68.0 ± 5.2	30.1 ± 1.6
Respiratory control ratio	3.4 ± 0.2	2.2 ± 0.1
ADP to oxygen ratio	1.73 ± 0.03	1.98 ± 0.07

[a] The reaction mixture contained succinate, 60 mM; Tris buffer, 25 mM; EDTA, 1 mM; KCl, 15 mM; MgCl, 25 mM; potassium phosphate, 15 mM; ADP, 0.36 mM; and DNP, 0.1 mM. The final volume of reaction mixture in the polarographic chamber was 2.0 ml at pH 7.3.

[b] Rate of respiration (mμmoles of O_2/mg of protein/min).

[c] Values (mean ± SEM) for 6 animals.

influenced by the availability of oxaloacetate, and the formation of OAA from succinate may be controlled by the NADH/NAD$^+$ ratio which represents the oxidation–reduction state in the mitochondria.

Succinate is oxidized preferentially over malate and fumarate in mitochondria and its rapid oxidation is known to produce a high ratio of NADH/NAD$^+$ through reverse electron transport (24). This inhibits conversion of malate to oxaloacetate.

Results in Table V show that PEP synthesis is very low from succinate after 10 min of incubation, while PEP synthesis is increased by 46-fold after 30 min of incubation.

Since pyruvate kinase (EC 2.7.1.40) activity is absent in guinea pig liver mitochondria (15), we have examined the possibility of PEP removal through reversal of PEPcK. Results in Table VI show that when guinea pig liver mitochondria were incubated in the presence of radioactive bicarbonate, there was significant incorporation of $^{14}CO_2$ into organic acid, mainly malate and citrate. This incorporation of radioactive $^{14}CO_2$ can be catalyzed through reversal of PEPcK. PEPcK also catalyzes an ITP-dependent oxaloacetate–$H^{14}CO_3^-$ exchange which is faster than either the decarboxylation or carboxylation. At pH 6.8, these respective rates of exchange, decarboxylation, and carboxylation of PEPcK are 30; 8.3; 1.0 (25). Malonate (6.6 mM) increased $^{14}CO_2$ incorporation by approximately 90%. In the presence of 5 mM PEP, malonate stimulated $^{14}CO_2$ incorporation by approximately 56% above that occurring without added PEP. Thus even though most of the $^{14}CO_2$ incor-

TABLE IV. Effect of 2,4-Dinitrophenol on Succinate Metabolism in the Presence of ADP in Guinea Pig Mitochondria.

Addition to system[a]	Metabolite content[b] (mμmoles/3.0 mg of N/30 min); found Malate	PEP	AcAc[c]	β-OH-butyrate[c]	Ratio β-OH-butyrate/AcAc)
None (4)	9.4 ± 0.2	3.3 ± 0.15	0.032 ± 0.013	0.166 ± 0.048	5.18
DNP, 0.033 mM (4)	6.5 ± 0.4 ($p < .01$)[d]	5.4 ± 0.30 ($p < .01$)[d]	0.073 ± 0.014	0.086 ± 0.007	1.18

[a] Reaction mixture contained 3.3 mM ADP, 13.3 mM $KHCO_3$, and 3.0 mg of mitochondrial nitrogen.

[b] Values (mean ± SEM) for 4 animals.

[c] AcAc = acetoacetate; β-OH-butyrate = β-hydroxybutyrate.

[d] p represents the probability of two values differing by chance determined by Student's t test.

TABLE V. Effect of Varying Incubation Time on Succinate Metabolism in Guinea Pig Liver Mitochondria.

Time incubated (min)	Metabolite content (μmoles/3.2 mg of N[a]); found	
	Malate	PEP
10	7.2	0.03
30	11.8	1.38

[a] Reaction mixture contained 3.3 m*M* ATP and 3.2 mg of mitochondrial nitrogen.

porated could be due to the exchange, the reported values for PEP synthesized under stimulated conditions (Tables I, II, and IV) may represent minimal values if simultaneous stimulation of the reversal of PEPcK occurs. This reverse reaction may be stimulated through the action of nucleosidediphosphate kinase and GTP:AMP phosphotransferase (EC 2.7.4.10). These enzymes can substantially reduce GTP concentrations when ADP and AMP levels are increased (15).

Fatty acid oxidation is known to increase production of NADH and thus causes an increase in the NADH/NAD$^+$ ratio. Results in Table VII show that L-octanoylcarnitine is also a potent inhibitor of PEP synthesis in the presence of ATP or ADP. As L-octanoylcarnitine concentrations were increased, the formation of β-hydroxybutyrate, total ketone bodies, and the ratio of β-hydroxybutyrate/acetoacetate were also increased. The degree of inhibition of PEP synthesis paralleled the degree of fatty acid oxidation and the ratio of β-hydroxybutyrate/acetoacetate. Results in Table VIII show that rotenone also inhibits PEP synthesis. Rotenone is known to inhibit oxidation of reduced pyridine nucleotides in the electron transport chain. This maintains a high NADH/NAD$^+$ ratio and inhibits OAA formation from malate.

Discussion. Succinate can be metabolized in guinea pig liver mitochondria to CO_2 and PEP by the known steps of the TCA cycle. Since oxaloacetate is the immediate precursor of PEP, its concentration is an important regulatory factor for PEP synthesis. Oxaloacetate formation can be influenced by uncouplers and phosphate acceptors which lower the mitochondrial NADH/NAD$^+$ ratio.

The ADP or ATP dependence of PEP stimulation seems to indicate that the formation of PEP is not controlled by substrate level phosphorylation in guinea pig liver mitochondria, as reported by Ishihara and Kikucki (8), but by the ATP/ADP ratio. This controls the GTP/GDP ratio through nucleosidediphosphate kinase (15).

Stimulation of PEP synthesis from succinate by DNP is probably due to stimulation of ATPase activity. This relieves the TCA cycle from control by phosphate acceptor levels and stimulates respiration. Increased respiration lowers the NADH/NAD$^+$ ratio, increases the conversion of malate to oxalo-

TABLE VI. Incorporation of (^{14}C) HCO_3^- into Organic Acids During Succinate Metabolism by Guinea Pig Liver Mitochondria.

Addition to system[a] (m*M*)	(μmoles/2.7 mg of N/25 min[b]) Metabolite content		
	Total $^{14}CO_2$ incorporated	^{14}C in	
		Malate	Citrate
None	0.58	0.43	0.05
Malonate, 6.6	1.13	0.11	0.27
Malonate, 6.6; PEP, 5	2.62	1.24	0.11
PEP, 5	0.84	—	—

[a] Reaction mixture contained (^{14}C) $KHCO_3$, 13.3 m*M*; 3.3 m*M* ATP; and 2.7 mg of mitochondrial nitrogen.

[b] Incubation time was 25 min.

TABLE VII. Effect of L-Octanoylcarnitine on Succinate Metabolism in the Presence of ADP or ATP, by Guinea Pig Liver Mitochondria.

Octanoylcarnitine (m*M*) added to system[a]	Metabolite content (μmoles/3.2 mg of N/30 min[b]) Found: Malate	PEP	β-OH-butyrate[c]	AcAc	Total ketones	Ratio (β-hydroxybutyrate/acetoacetate)
With 3.3 m*M* ADP,						
0	9.3	3.85	Trace	Trace	Trace	—
0.33	10.7	1.25	0.43	0.59	1.02	0.7
0.67	11.6	0.15	1.68	0.47	2.15	4.3
1.33	13.0	Trace	2.25	0.50	2.75	4.3
With 3.3 m*M* ATP,						
0	9.0	2.10	0.19	Trace	0.26	—
0.33	12.3	0.67	0.74	0.37	1.11	2.0
0.67	13.1	0.07	1.56	0.46	2.02	3.9
1.33	13.5	Trace	1.45	0.50	1.95	5.1

[a] Reaction mixture contained 13.3 m*M* $KHCO_3$ and 3.2 mg of mitochondrial nitrogen.
[b] Incubation time was 30 min.
[c] β-OH-Butyrate = β-hydroxybutyrate; AcAc = acetoacetate.

acetate, and thus increases PEP formation. The inhibition of PEP synthesis by fatty acids or rotenone is due to the increase in the NADH/NAD^+ ratio. This decreases the conversion of malate to oxaloacetate, as evident by increased accumulation of malate (Table VI), and inhibits PEP synthesis.

The increase in the PEP formation by malonate is attributed to a decreased metabolism of succinate. The decreased substrate flow through succinate dehydrogenase, while still providing sufficient malate for possible conversion to PEP, allows the cytochromes to become more oxidized. The lowered NADH/NAD^+ ratio then increases the conversion of malate to oxaloacetate. The expected low value of malate in the presence of malonate is confirmed in Table II.

It can be concluded that guinea pig liver mitochondria in the absence of substrate level phosphorylation can synthesize PEP from succinate at a significant rate. The synthesis of PEP from succinate is controlled by the concentration ratios of intramitochondri-

TABLE VIII. Effect of Rotenone and Palmitoylcarnitine (PC) on Succinate Metabolism in the Presence of ADP in Guinea Pig Liver Mitochondria.

Addition to system[a] (m*M*)	Metabolite content[c] (μmoles/3.2 mg of N/30 min[b]); found: Malate	PEP	AcAc[d]	β-OH-butyrate[d]
None	11.31 ± 0.31	2.16 ± 0.32	—	—
0.67 PC	12.87 ± 0.33	0.88 ± 0.22	0.28 ± 0.04	0.52 ± 0.08
0.20 Rotenone	14.35 ± 0.56	Trace	—	—
0.67 PC 0.20 rotenone	14.81 ± 0.23	Trace	Trace	0.06 ± 0.03

[a] Reaction mixture also contained 3.3 m*M* ADP, 13.3 m*M* $KHCO_3$; and 3.2 mg of mitochondrial nitrogen.
[b] Incubation time was 30 min.
[c] Values (mean ± SEM) for 4 animals in duplicate.
[d] AcAc = acetoacetate; β-OH-butyrate = β-hydroxybutyrate.

al pools of NADH/NAD$^+$ and ATP/ADP, and that under our conditions, the availability of these cofactors, at least in state 4, is rate limiting for PEP synthesis.

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