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The Use of a Continuous Assay System to Show the Stimulation of Phosphoenolpyruvate Production in Intact Mitochondria by Valinomycin and by Mn^{2+}

M. Granger and E. J. Harris

Departments of Biochemistry and Biophysics,
University College
London, WC1E 6BT, England

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Abstract

1. A method for the direct recording of the PEP efflux from isolated mitochondria is described.
2. This method has been used to show the stimulation of PEP efflux by externally added Mn^{++} ions.
3. Valinomycin, uncoupler and oleate were also shown to stimulate PEP efflux.
4. Valinomycin caused an increase in the internal concentration of both PEP and citrate.
5. The results indicate that the major pathway of PEP synthesis in isolated mitochondria is via PEP carboxykinase and the results do not call for an unknown pathway of metabolism.
6. Two interactions between PEP and citrate are described; competition for the mitochondrial interior and the stimulation of PEP production by citrate.

Abbreviations
EGTA: ethylene glycol-bis(2-aminoethyl)-tetraacetate.
TTFB: tetrachlortrifluoromethylbenzimidazole.

Enzymes
Pyruvate Kinase: EC 2.7.1.40
Lactate Dehydrogenase: EC 1.1.1.27
Pyruvate Carboxy Kinase: EC 4.1.1.32

Introduction

The key reaction in gluconeogenesis is the production of phosphoenolpyruvate from oxaloacetate by reaction:

$$OAA + XTP \leftrightarrow PEP + XDP + CO_2$$

In mitochondria X is guanosine. The enzyme concerned is pyruvate carboxykinase (E.C.4.1.1.32). The activity of the enzyme depends on the dietary state.[1] It is normally stimulated by Mg^{2+} ions but Mn^{2+} ions will replace them and will increase the maximal activity obtained with Mg^{+} alone.[2] The distribution of the enzyme between cytosol and mitochondria varies with the species. Rat-liver mitochondria are relatively poorly

endowed with it[3]; however, conditions can be established under which there is an appreciable production of PEP from either oxoglutarate,[4] or malate.[5]

To study the process as it occurs in isolated mitochondria it is convenient to record directly the PEP output by the use of a suitable reaction in the suspension medium. A method for doing this has been devised and used to show the stimulation of the enzyme in intact mitochondria by externally added Mn^{2+}. A condition which favours the production of PEP is the loading of the mitochondria with substrate at the same time as the redox state is driven oxidized by an energy demand. This condition is obtained when an ionophore such as valinomycin is used to stimulate the energy requiring uptake of K^+, since substrate is also carried in.

Although export of PEP from the mitochondria to the cytosol is required either for reaction with pyruvate kinase to form ATP or with enolase to form 2-phosphoglycerate it is of interest that the compound may be accumulated to give a high inside/outside concentration ratio, approaching that which holds for citrate.

Methods

Liver mitochondria were prepared by a slight modification of Schneider's method[6] using 0·5 mM EGTA and 0·05% dialyzed bovine serum albumin in the 0·25 M sucrose homogenization medium and in the 0·3 M sucrose wash medium.

Kinetic Recording. The mitochondria were suspended at approximately 0·5 mg protein/ml, in each of two 3 ml cuvettes in the two paths of a Pye Unicam SP1800 double-beam recording spectrophotometer. Assays were carried out at 25°C in a medium having sucrose 250 mM; KCl 5 mM; $MgCl_2$ 5 mM; Tris-chloride pH 7·4, 20 mM. For the continuous assay of PEP the following were added to both cuvettes: ADP to 100 μM, NADH to 100 μM, atractylate, 10 μg, and 5 units of dialyzed lactate dehydrogenase (E.C.1.1.1.27). The atractylate prevents phosphorylation of the externally added ADP by the mitochondrial respiratory chain. To one cuvette (the "reaction cuvette") was also added 0·5 units of dialyzed pyruvate kinase (E.C.2.7.1.40). The enzymes were dialyzed to remove ammonium ions. The reaction was started by the addition of substrate to both cuvettes. All subsequent additions were also made to both cuvettes. In the "reaction cuvette", the PEP leaving the mitochondria phosphorylates the ADP as it is converted to pyruvate and the pyruvate formed is reduced to lactate at the expense of NADH, thus causing a fall in absorption. Direct production of pyruvate, presumably from oxalacetate, occurs on both sides and cancels out in the differential output. In some experiments direct records of pyruvate production were obtained by having lactate dehydrogenase without pyruvate kinase in the "reaction cuvette". In a system having oxoglutarate 1 mM; malate 1 mM; phosphate 1 mM, the rate of pyruvate production was approximately 50% of the rate of PEP production. It was confirmed that when LDH was added to each cuvette, without pyruvate kinase in either, the two sides remained balanced. Figure 1 illustrates a recording obtained for PEP production in an experiment in which the stimulation by TTFB was measured. Provided care was taken in the mitochondrial preparation there was negligible spontaneous NADH oxidation.

The swelling of the mitochondria in the cuvettes can reduce the optical density and it is therefore important to check such changes for any possible inequality by taking

readings at 700 nm. This was done in all the experiments. In most experiments volume changes were small and equal so they cancelled out in the differential output. In the valinomycin experiments, such a large volume change occurred as to cause a discrepancy

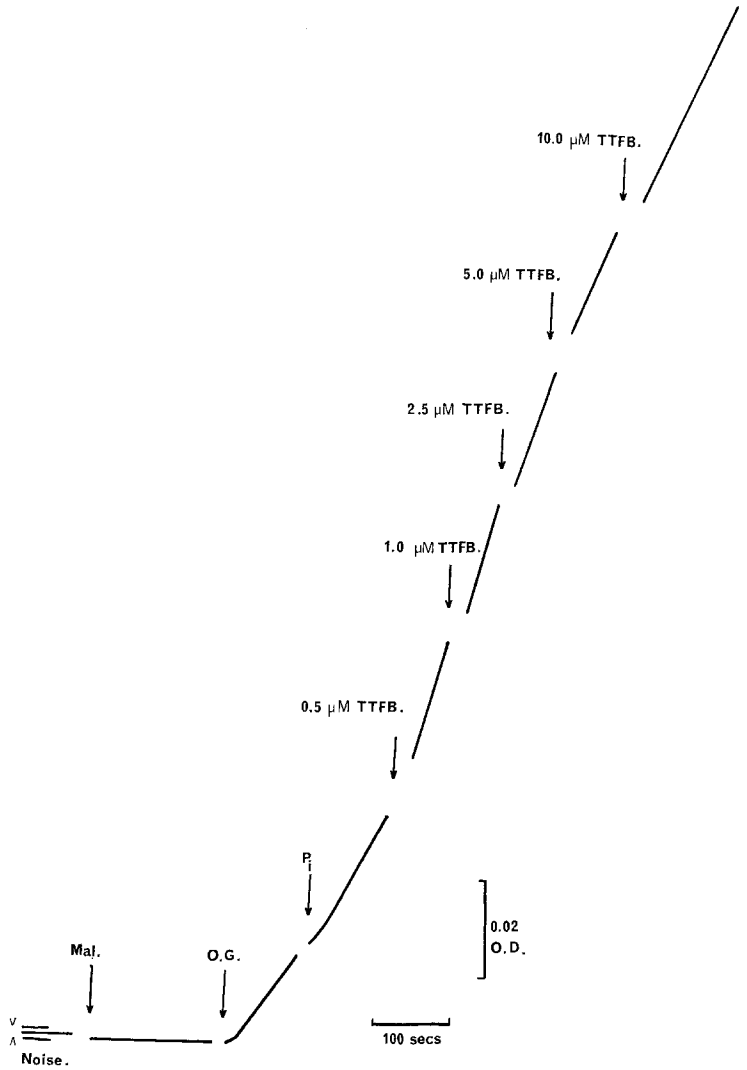

Figure 1. Tracing from continuous recording of the production of PEP from rat liver mitochondria. There is no rate from malate as substrate, oxoglutarate addition causes the reaction to start. Titration in of the uncoupler tetrachlortrifluoromethylbenzimidazole (TTFB) leads to stimulation of the rate. For details of medium see in "Methods". Additions as in Table 1B.

in the differential output and it was then necessary to wait about 1 min until the volume became constant before measuring the rate of PEP production.

The distribution of PEP between mitochondria and medium was found by taking samples from incubations and separating the mitochondria by centrifugation through

silicone as described before.[7] These incubations were carried out in a medium having KCl 120 mM; $NaHCO_3$ 20 mM; mannitol 60 mM; $MgCl_2$ 2 mM; equilibrated with 95% O_2 + 5% CO_2. This medium was the most convenient for experiments involving centrifugation through silicone. Its use in the kinetic recording method gave results similar to those presented. Substrate and phosphate were added as tris salts. After centrifugation the supernatant above the silicone and the acid extract of the mitochondria beneath were assayed for citrate and PEP by fluorimetric methods.[8]

Results

(*Note:* evidence that the method duly cancels changes of absorbance due to swelling or pyruvate production is given in "Methods".)

Stimulation of PEP Production by Valinomycin or Mn^{2+}

Under the conditions employed there was no measurable PEP production from malate with, or without, added phosphate. Addition of valinomycin initiated the efflux of PEP and further addition of oxoglutarate more than doubled the rate (Table 1A). When uncoupler is added after valinomycin[7] it reduces the rate (Table 1A, line 5).

A significant rate of PEP production was obtained when oxoglutarate was the only added substrate (Table 1B, line 1). The rate was little, if at all, increased by malate (line 2) but phosphate caused an increase in rate (line 3). Phosphate is required to phosphorylate GDP and is removed from the system as PEP.[4]

A Mn^{2+} salt added in the presence of malate and oxoglutarate stimulated PEP production. The increase in rate passed through a maximum as the quantity of Mn^{2+} was increased. Figure 2 shows the [Mn^{2+}]-rate relation. Added Mn^{2+} is known to be accumulated in the mitochondria[9] so that little remains in the medium. Measurements were made of the Mn^{2+} in the supernatant when an amount corresponding to 10 μM had been added to a suspension which had 7·5 mg protein/ml. The concentration in the medium was only 5 μM so that the mitochondria had taken up 0·66 μmole/g protein at this applied concentration.

With citrate as substrate there was no PEP production until valinomycin was added (Table 1C, line 2). In this situation fluorocitrate inhibited the process (Table 1C, line 3). Isocitrate relieved the inhibition (see 1D, line 4) showing that fluorocitrate was acting by blocking the aconitase step. Fluorocitrate is presumably more inhibitory in the presence of valinomycin since this condition will tend to favour the loading of the mitochondria with fluorocitrate. Addition of malonate again (1D, lines 5, 6) stopped PEP production by inhibiting the oxidation of succinate which provides oxaloacetate. This inhibition could be relieved by the addition of malate (1D, line 7), since the succinate thiokinase step still provides GTP. The action of malonate on a system having only oxoglutarate is shown in Table 1E.

Stimulation by Uncoupler and by Oleate

It has been shown, using rabbit liver mitochondria, that a limited degree of uncoupling stimulates the rate of PEP production. This stimulation passes through a maximum as the uncoupler is titrated in.[10] Our method was particularly suitable for the titration

TABLE I. Rates of PEP efflux rat liver mitochondria found in the continuous assay system which holds the external concentration near to zero. Temperature 25°C. For basic reaction mixture see "Methods"

A. Stimulation of PEP production from malate by valinomycin and by oxoglutarate

Serial Addition	Concn. of additive	Rate of PEP production nmole/mg/min
1 Malate	1 mM	0·00
2 Phosphate	0.5 mM	0·00
3 Valinomycin	66 ng/mg	2·32
4 Oxoglutarate	1 mM	5·17
5 TTFB	1 μM	2·04

B. Stimulation of PEP production from oxoglutarate by malate, phosphate and Mn^{++} (see also Fig. 2)

Serial Addition	Concn. of additive	Rate of PEP production nmole/mg/min
1 Oxoglutarate	1 mM	0·82
2 Malate	1 mM	0·95
3 Phosphate	1 mM	1·29
4 $MnCl_2$	10 μM	1·97

C. Stimulation of PEP production from citrate by valinomycin, abolition of production by fluorocitrate

Serial Addition	Concn. of additive	Rate of PEP production nmole/mg/min
1 Citrate	1 mM	0·00
2 Valinomycin	66 ng/mg	9·25
3 Fluorocitrate	10 μM	0·00

D. Stimulation of PEP production from citrate by Mn^{++}, inhibition by fluorocitrate, relief by isocitrate, further inhibition by malonate and relief by malate

Serial Addition	Concn. of additive	Rate of PEP production nmole/mg/min
1 Citrate	1 mM	0·00
2 $MnCl_2$	100 μM	1·12
3 Fluorocitrate	10 μM	0·74
4 Isocitrate	1 mM	1·24
5 Malonate	1 mM	0.50
6 Malonate	5 mM	0·00
7 Malate 1mM + P_i	0·5 mM	1·24

E. Inhibition of PEP production from oxoglutarate by malonate and relief by malate

Serial Addition	Concn. of additive	Rate of PEP production nmole/mg/min
1 Oxoglutarate	1 mM	0·74
2 Malonate	10 mM	0·00
3 Malate 1mM + P_i	0·5 mM	0·74

procedure and Fig. 3 shows the stimulation passing through a maximum as the uncoupler TTFB was titrated into a suspension of rat liver mitochondria.

Oleate at 30 μM had a similar effect to the uncoupler and stimulated PEP formation by a factor of 2. That the effect was essentially attributable to uncoupling was shown by the fact that if bovine serum albumin at 1mg/ml had first been added the oleate no longer had any effect.

Intramitochondrial Accumulation of PEP Under the Influence of Valinomycin.

Valinomycin induces the uptake of K^+ with such anions as penetrate the mitochondrial matrix.[11] The generation of an anion intramitochondrially will lead to a

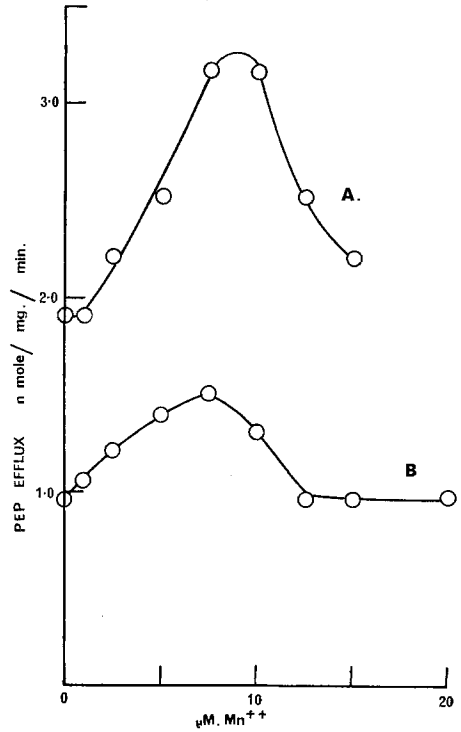

Figure 2. Stimulation of the rate of PEP production by mitochondria when a Mn^{2+} salt is titrated in. Curve A was obtained with material from rabbit liver and curve B with material from rat liver. Malate 1 mM, oxoglutarate 1 mM and phosphate 0·5 mM were present, other conditions as described in "Methods".

tendency for its internal concentration to rise to a new level at which the rate of export equals the rate of production. Assays of PEP and citrate contents of rat liver mitochondria were made before and after addition of valinomycin with malate as substrate. Figure 4A and B shows the results. In both species the ionophore caused an increase in the contents of both anions. In this condition there was no consumption of PEP (because there was no pyruvate kinase added) and its distribution between mitochondria and medium was similar to that of citrate though the inside/outside ratio was not so high.

Interrelations Between PEP and Citrate

Since PEP and citrate share the matrix space it was of interest to examine the consequences of changes in one compound upon the other. Production of PEP from citrate was shown with valinomycin present (Table 1c). There is however the additional like-

lihood of displacements occurring; Gamble and Mazur[16] noted that citrate addition drove out mitochondrial PEP and phosphate. Assays were made of PEP and citrate in experiments in which first, the conditions were altered to produce one or both substances, and then one of the substances was added to the medium. Figure 5A shows the results obtained with pyruvate as substrate. Addition of malate temporarily increased the content of PEP and displaced some of the citrate. Later, citrate formation tends to restore the internal level which is again lowered when exogenous PEP is added. Figure 5B shows results obtained with malate as substrate. Palmitoyl carnitine caused a rapid increase of citrate and a slow rise of PEP. The latter effect is likely to be due to the citrate providing oxoglutarate, and hence GTP, to react with oxaloacetate. In contrast, adding

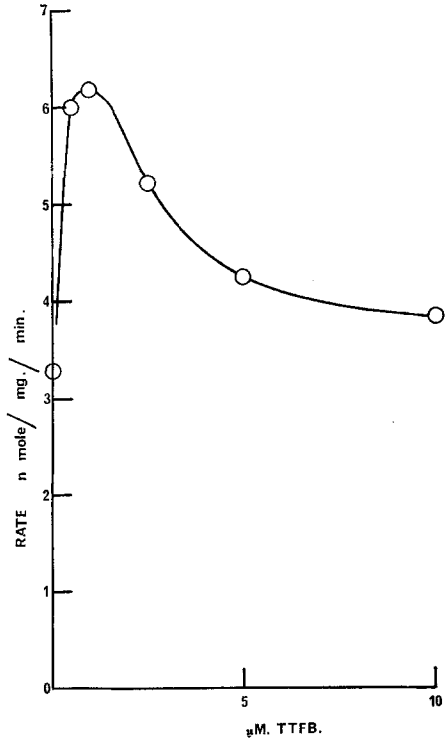

Figure 3. Stimulation of PEP production by mitochondria (rat liver) by TTFB (see Fig. 1). Malate 1 mM, oxoglutarate 1 mM and phosphate 0·5 mM were present, other conditions as described in "Methods".

a relatively high concentration of exogenous citrate caused a fall in PEP content. The ratios between mitochondrial content and external concentration found for citrate and PEP in these experiments are shown in Table 2.

Discussion

The results obtained can be conveniently discussed with reference to the following reactions:

(1) OAA + GTP $\leftrightarrow$ PEP + GTP
(2) Oxoglutarate + NAD $\leftrightarrow$ Succinyl CoA + NADH
(3) Succinyl CoA + GDP $\leftrightarrow$ Succinate + GTP + CoA
(4) Succinate $\leftrightarrow$ (Fumarate) $\leftrightarrow$ Malate

(5) Malate + NAD $\leftrightarrow$ OAA + NADH
(6) NADH + O_2 (respiratory chain) + ADP + Pi $\leftrightarrow$ NAD + ATP
(7) ATP + GDP $\leftrightarrow$ ADP + GTP

With oxoglutarate alone reactions 2, 3, 4, and 5 provide the reactants for reaction 1. When the mitochondrial content of oxoglutarate and Pi is raised by valinomycin[11]

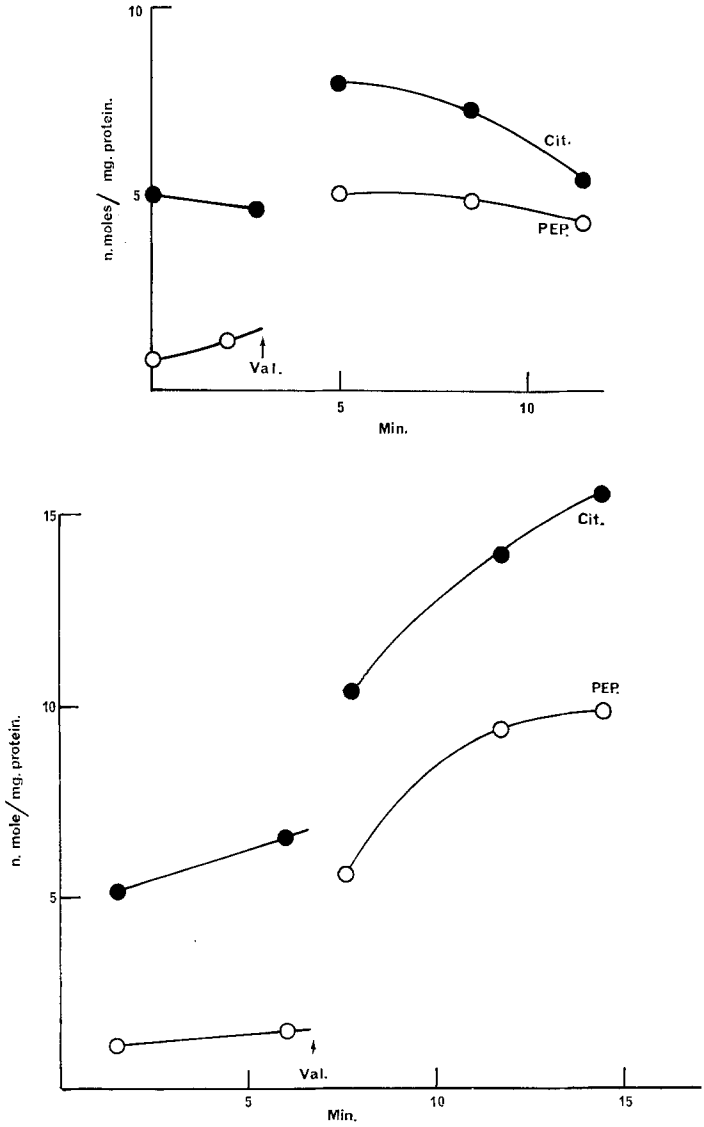

Figure 4. A. Response of mitochondrial contents of citrate and PEP caused by valinomycin at 30 ng/mg protein applied to a rat liver preparation. Protein 7 mg/ml.

B. The same for rabbit liver mitochondria. The media contained: sucrose 250 mM; KCl 5 mM; $MnCl_2$ 16 M; tris phosphate 0·5 mM; tris malate 2·5 mM; fluorocitrate 16 μM; tris chloride pH 7·4 20 mM.

and NADH is removed by oxidation to energize the induced K^+ uptake, the rate of reaction 1 is increased, partly due to reaction 5 being driven towards the right. With malate alone our data indicate that the amount of OAA formed by reaction 5 and such GTP formation as occurs due to 6 and 7 operating is insufficient to give a measurable PEP production. When valinomycin is added the stimulated respiration and uptake of malate combine to drive 5 to the right and reactions 6 and 7 evidently operate to provide sufficient GTP. Experimental evidence for this is provided by the fact that oligomycin stops PEP production from malate in the presence of valinomycin; PEP production is

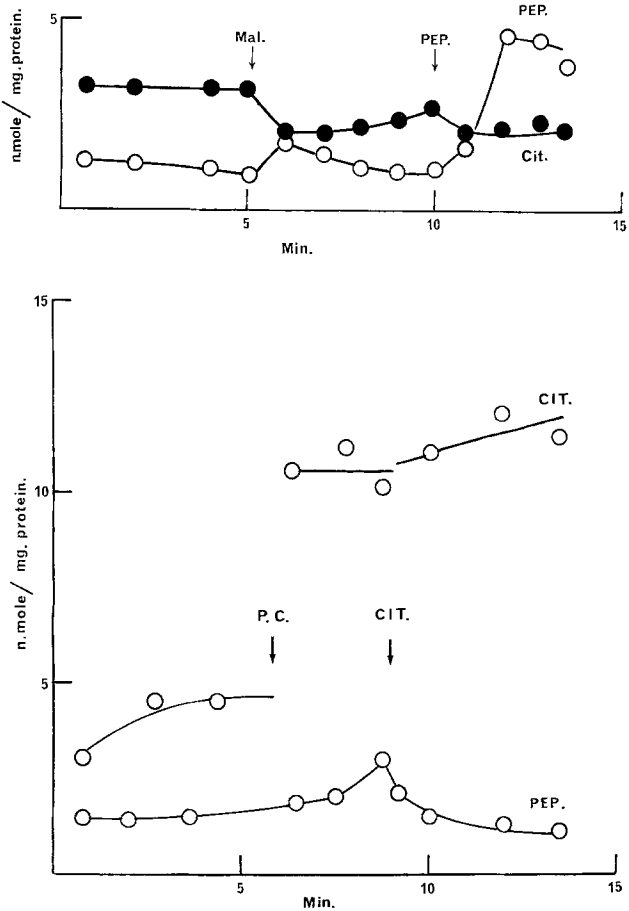

Figure 5. A. Displacements of intramitochondrial citrate in response to additions to the medium of (1) malate (150 μM) and (2) phosphoenolpyruvate (166 μM initially). Note the displacement of internal PEP by the citrate generated between the 6th and 10th minutes. The medium contained: pyruvate 1 mM; sucrose 150 mM; KCL 60 mM; tris chloride pH 7·4 10 mM; tris phosphate 6 mM; $MnCl_2$ 65 μM. Protein 9·3 mg/ml.

B. Stimulation by palmitoyl carnitine (25 μM) of the accumulation of both PEP and citrate when malate is present. Displacement of PEP by added citrate (25 μM).

The medium was as in 5A except that malate 1 mM replaced pyruvate.

restored by the addition of oxoglutarate or citrate since reaction 2 then provides the GTP. Restoration by citrate is abolished by fluorocitrate since this blocks aconitase which converts citrate to isocitrate. Isocitrate restores PEP production even in the presence of fluorocitrate.

TABLE II. Accumulation of PEP and citrate in rat liver mitochondria. The ratios are of content (μmole/g protein corrected for the amount carried down in the sucrose accessible space) over the concentration in mM

See Fig. 5A.	Ratios		*See Fig. 5B.*	Ratios	
Conditions	*PEP*	*Citrate*	Conditions	*PEP*	*Citrate*
Incubation with pyruvate	40	50	Incubation with malate	45	73
Same after malate addition	32	59	Same after palmitoyl carnitine addn.	110	80
Same after PEP addition	43	35	Same after citrate addition	33	8·5

Malate alone is a slowly utilized substrate and the level of internal ATP is low, which does not favour GTP production. Presumably it is for this reason that the addition of oxoglutarate to a system containing malate initiates PEP formation by providing GTP from reactions 2 and 3. This process does not require ATP and is found, experimentally, to be insensitive to oligomycin. Addition of uncoupler also stimulates PEP production by driving reaction 5 to the right. Excess uncoupler probably leads to a reduction in PEP production for one of the reasons that it also leads to a fall off in respiration, namely a discharge of substrate and phosphate.[12, 13]

The results presented do not call for an unknown pathway of metabolism. The stimulation by Mn^{2+} in the intact particles accords with the known stimulation in vitro of PEP carboxykinase by this cation[2] and presumably represents a stimulation additional to that provided by the endogenous Mg^{2+} present in the mitochondria. The additive action of the two cations has been described.[14] Bartley and Dean[5] argued that the absence of stimulation by added Mg^{2+} pointed to another process occurring. However, there seems no reason to ignore the endogenous Mg^{2+}, of which only a small amount of the total mitochondrial content of 25–33 μmole/g protein need be ionized to provide adequate stimulation. The same authors' argument that externally added GTP fails to stimulate PEP production can be countered by questioning whether this nucleotide has access to the enzyme. Heldt[15] has shown that the external guanine nucleotides do not directly communicate with the endogenous guanine nucleotides.

Two interactions are described in this paper between citrate and PEP. There is a competition between them for the mitochondrial interior as seen in Fig. 5 and there is stimulation of PEP production by citrate. The latter has been shown to be due to the conversion of citrate *via* isocitrate to oxoglutarate which furnishes GTP for the PEP forming reaction.

Acknowledgements

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