STIMULATION OF MITOCHONDRIAL PYRUVATE CARBOXYLATION BY Mn^{2+} AND THE DISTRIBUTION OF THE PRODUCTS BETWEEN MITOCHONDRION AND MEDIUM

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1. Introduction

The CO_2 formed by the oxidative decarboxylation of pyruvate in mitochondria can be used to carboxylate more pyruvate to oxalacetate. The latter compound either condenses with the acetyl CoA formed as one of the decarboxylation products or is reduced to malate [1,2]. A third path, mediated by GTP, leads from oxalacetate to phosphoenolpyruvate with release of CO_2; this process requires a supply of phosphate [3] and is stimulated by long chain fatty acids or uncouplers [4], otherwise it is slow. Mehlman, Walter and Lardy [5], showed that the main products containing ^{14}C when mitochondria were incubated with pyruvate and ^{14}C-bicarbonate, were malate and citrate with fumarate equal to about 20% of the malate. They, as well as Stuart and Williams [2], noted that some unlabelled precursor, suggested to be fatty acid, also fed carbon into the cycle. It seems valid to regard the increments in malate and citrate, in the presence of fluorocitrate to block the aconitase, as a good measure of the pyruvate carboxylated when uncoupler is not added, and the experiment avoids ambiguity about the source of the carbon.

The products distribute between the mitochondria and the medium with high internal/external ratios [6–8], and there is competition between the anions to neutralise the mitochondrial cations. It is important to know the distribution, because in the intact cell it is the cytoplasmic concentration of citrate which is one of the controls of phosphofructokinase and acetyl CoA carboxylase. This paper presents values for the distribution and shows that Mn^{2+} stimulates mitochondrial pyruvate carboxylation and the isolated enzyme prepared from rat liver. Utter and Keech [9] described the stimulating effect of Mn^{2+} on the enzyme derived from chicken liver. We have now shown that Mn^{2+} offsets the inhibitory effect of Ca^{2+} as suggested before [10].

2. Methods

Rat liver mitochondria were prepared as described previously [11]. The mitochondria were suspended at 50 mg/ml in 0.25 M sucrose. The incubations were carried out in medium containing KCl 120 mM, $NaHCO_3$ 20 mM, $MgSO_4$ 0.67 mM (except where stated), tris pyruvate 2 mM, fluorocitrate 10 μM and trace quantities of ^{14}C sucrose. Before use the solution was gassed with 95% O_2, and 5% CO_2, and during the incubation a stream of the same mixture was played on the surface of the suspension. The protein content was between 7 and 8 mg/ml and the temperature 21 ± 1°. Samples of 0.2 ml were taken from the incubation and put into small plastic centrifuge tubes preloaded with silicone (G.E. type F.50) over 40 μl 1.5 M perchloric acid. The tubes were centrifuged in Coleman 'Microfuges' as described by Harris and Van Dam [12]. Centred on selected times, sets of three samples were taken (over about 1 min total interval) to provide material for enzymatic assays and radioactivity and manganese measurements. The medium remaining on top of the silicone was promptly acidified and used to find the concentrations in the medium, the acid extracts of the mitochondrial pellets were recovered from beneath the silicone. The distribution of

the radioactivity allowed correction to be made for the medium carried down in the total sucrose accessible space of mitochondria and adherent fluid.

Fluorimetric assays for malate were made using malate dehydrogenase, acetyl CoA, NAD and citrate synthase as described by Palmieri and Quagliariello [13]. Consecutive assays for pyruvate and (sometimes) phosphoenolpyruvate were made, using lactate dehydrogenase and pyruvate kinase, followed by the assay for citrate using citrate lyase in presence of malate dehydrogenase and excess NADH. Manganese was measured by atomic absorption spectrometry.

Pyruvate carboxylase was prepared from a washed mitochondrial pellet (about 10 mg protein) by suspending it in water to remove sucrose and salts, followed by centrifugation at 100,000 × *g*/min followed by resuspension in water. The suspension was freeze-dried. The solid matter was extracted in 5 ml 125 mM tris chloride at pH 7.4 at room temperature and removed by centrifugation at 200,000 × *g*/min. The supernatant was brought to 16% saturation with ammonium sulphate and recentrifuged, the precipitate was rejected and the ammonium sulphate concentration was raised to 38%. The resulting precipitate was recovered by centrifugation and was finally dissolved in about 5 ml 50% by wt sucrose at 30°. The pyruvate carboxylase activity was assayed by a spectrophotometric method [9].

3. Results

3.1. *The medium*

The KCl-$NaHCO_3$ medium was used because it more closely approximates cytoplasm than a tris-buffered medium. A comparison between the two media was made and it showed that the internal citrate contents reached with no stimulation by Mn was about 20% higher in bicarbonate than in tris. For example, in 5 min in the tris mixture the citrate was 4.5 μmole/g protein and it was 5.5 μmole/g in the bicarbonate. A more important difference appeared between the respective citrate concentrations in the media which were 35–40 μM in the tris mixture and 60–75 μM in the bicarbonate. The malate contents and concentration showed no significant differences.

A set of measurements of the internal/external distribution ratios of dimethyl oxazolidinedione were made in each medium. These were used to estimate the internal pH according to the method used by Addanki, Cahill and Sotos [14]. The weak acid was relatively less concentrated in the mitochondria when the bicarbonate buffer was used; it was calculated that the internal pH was 0.1 unit more acid than with the tris buffer.

3.2. *Stimulation of citrate formation by Mn^{2+}*

It was previously shown that the uptake of Ca led to an increase mitochondrial content of citrate provided pyruvate and malate, either endogenous or added, were present [10,15]. If Mn^{2+} is added in presence of pyruvate there is a prolonged formation of citrate without depletion of the endogenous malate. There is evidently a production of oxalacetate and hence of citrate from the pyruvate, the difference is doubtless connected with the fact that Ca^{2+} inhibits pyruvate carboxylase [16], as suggested by Gevers and Krebs [17].

Fig. 1 shows the results for citrate contents obtained in five incubations. Without Mn^{2+} addition but with Mg^{2+} present at 0.67 mM there was a slow citrate formation in the experiment plotted in the curve marked A of fig. 1, and less in the set marked A′, obtained with a different mitochondrial preparation. With Mn^{2+} added sufficient to make the mitochondrial content 24 μmole/g protein, with about 6 μM in the medium (as determined by the flame spectrophotometer) there is a rate of net citrate formation about 1.7 × that of curve A (curve B). With the same Mn^{2+} addition, but with Mg^{2+} at 0.67 mM as well, the rate (curve C) is about 4.5 × that of curve A, being now 1.3 μmole per g per min. The same rate could also be obtained by using more Mn^{2+} (sufficient to make the content 72 μmole/g protein) without Mg^{2+} (curve D). Note that in the last experiment the Mn was only added after the 10 min reading. Analyses made of supernatants recovered from the incubations showed that the citrate concentration (table 1) tended to follow the mitochondrial content, but internal retention of citrate was favoured by Mn^{2+}, so the ratio mitochondrial content in μmole/g: concentration was about 30 times in one of the controls, but was 200 times in presence of the higher Mn content or the Mn^{2+} + Mg^{2+} mixture. If, following Harris and Van Dam [11] and Pfaff et al. [18], the matrix space is regarded as the volume of water exchanged with tritiated water but not penetrated by sucrose, which is commonly between 0.7 and 1 ml/g protein, the results would mean that concen-

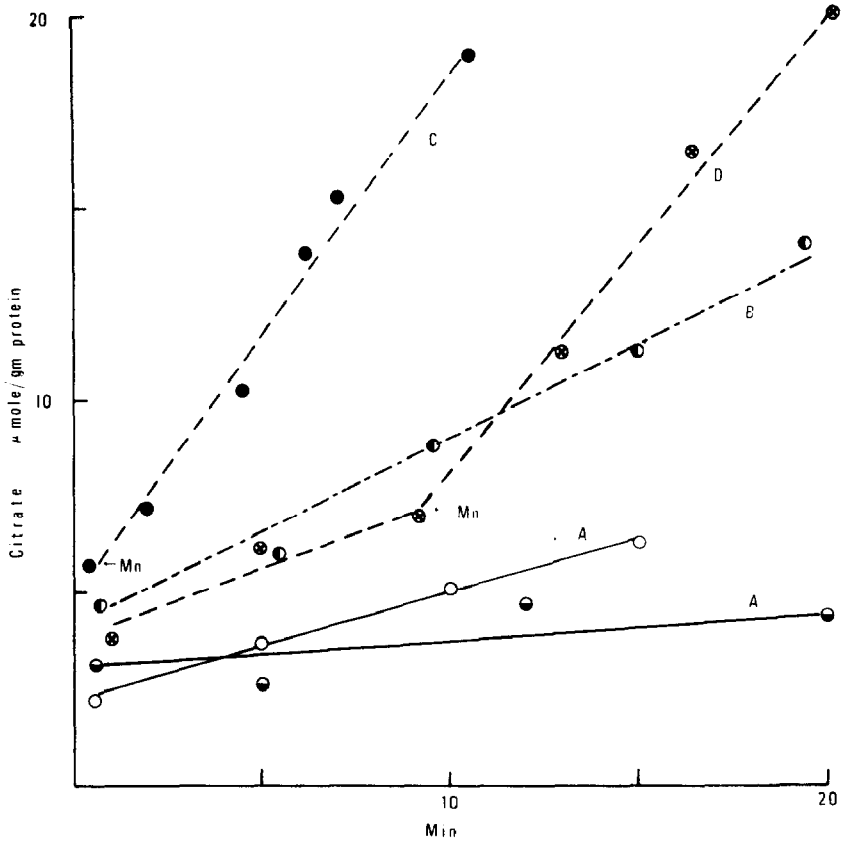

Fig. 1. Mitochondrial accumulation of citrate during incubations with pyruvate and the stimulatory effect of Mn. Temperature 21°. A: with Mg at 0.67 mM, no Mn. A': with Mg at 0.67 mM, no Mn. B: Mn added to give content 24 μmole/g, no Mg. C: Mn added to give content 25 μmole/g, Mg present at 0.67 mM. D: Mn added at 10 min to give content 72 μmole/g, no Mg.

tration gradients of citrate are between 30 and 200. Analyses for malate indicated that the mitochondrial content was fairly constant (table 1) while the concentration in the medium, though consistent within an experiment, could be as low as 5 μM or as high as 50 μM.

In an experiment made in parallel with run B it was noted that addition of *l*-palmitoyl carnitine at 15 μM increased the malate in the medium from 5 μM to 25 μM so it is suggested that the malate concentration factor may depend on the presence or absence of amphiphilic substances. This has to be explored further.

In some of the experiments the contents of mitochondrial phosphoenolpyruvate were measured; these remained static at levels of 1, 1.5 or 2 μmole/g protein. This substance is one of the inhibitors of pyruvate carboxylase, having a K_i of about 0.5 mM with respect to acetyl CoA, with which it is a competitor (J.Wimhurst and K.L.Manchester, unpublished).

When Mn was added to the medium, most of it was taken up by the particles: just after the addition in experiment B the concentration in the medium was only 6 μM and in 20 min it fell to 4 μM. The protein contained 24 μmole Mn^{2+}/g. In experiment D (fig.1 and table 1) the final Mn content of the particles was 72 μmole/g protein. With still more Mn, sufficient to make the content 90 μmole/g, the carboxylation process was inhibited.

In agreement with the authors quoted in the introduction, there was no citrate formation if the medium was supplemented with ADP and phosphate, or with a glucose-hexokinase trap. Mitochondrial pyruvate was concentrated with respect to the medium

Table 1
Values of mitochondrial malate content and malate and citrate concentrations in the medium during the incubations plotted in fig. 1.

Time	Run	Malate (μmole/g)	Malate (μM)	Citrate (μM)
	A′	1.4	40	71
	A	0.9	14	15
1 min	B (+Mn 24 nm/mg)	1.5	3	76
	C	2.0	27	29
	D	0.6	< 5	74
	A′	3.3	48	130
	A	0.8	30	35
5 min	B	2.0	4	96
	C (+Mn 25 nm/mg)	2.0	30	73
	D	1.3	< 5	92
	A′	2.5	53	181
	A	1.5	57	45
10 min	B	2.0	11	134
	C	2.0	< 5	97
	D	0.8	< 5	95
	A′	2.5	31	174
	A (+ PC 100 μM)	2.4	110	66
15 min	B	2.1	6	153
	C	–	–	–
	D (+Mn 72 nm/mg)	0.8	< 5	96

Medium: See section 2. PC ≡ *l*-palmitoyl carnitine.

Table 2
Mitochondrial accumulation of pyruvate in the incubations.

Run	External concentration (mM)		Content, μmole/g corrected for sucrose space	
	Intitial	Final	Initial	Final
A′	1.93	1.80	9.0	7.2
B	1.62	1.45	16.4	15.6
C	1.89	1.53	7.3	6.0
D	1.62	1.39	4.9	4.0

Medium: See section 2.

(table 2). In the cellular context this may be important, because this substrate will be applied to the mitochondrial enzymes at a higher concentration than that prevailing in the cytoplasm.

3.3. *Stimulation of pyruvate carboxylase activity by Mn^{2+}*

The previous experiments provided a clear indication that intramitochondrial Mn stimulates pyruvate carboxylation. Tests were made using the enzyme prepared from rat liver mitochondria. The activity of the carboxylase is plotted in fig. 2 with alternatively Mg^{2+} and Mn^{2+} as activating ions. Tests made with Mn added to a system having 8 mM Mg^{2+} showed that there was no appreciable change from the maximum activity (0.4

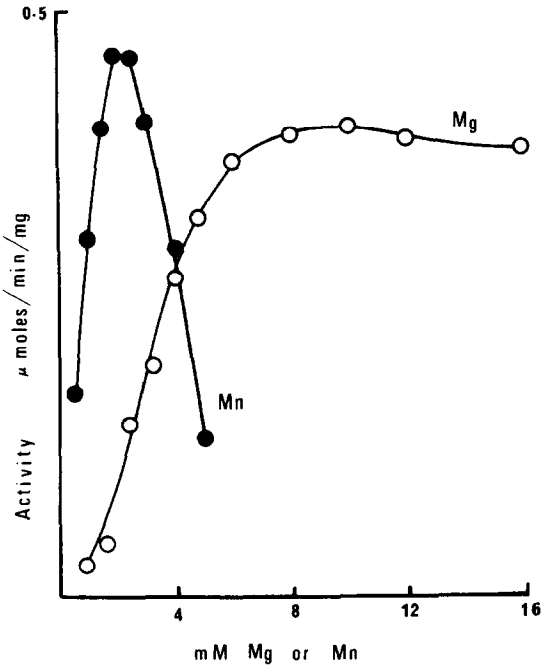

Fig. 2. Pyruvate carboxylase activity of an enzyme preparation from rat liver as a function of Mg^{2+} (o) or Mn^{2+} (•) concentration added to test medium. The ATP concentration was 2 mM. Temperature 30°.

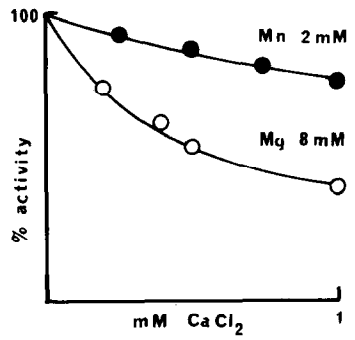

Fig. 3. Diminution of the pyruvate carboxylase activity of an enzyme preparation as Ca^{2+} is titrated into the test medium either with Mg^{2+} at 8 mM (o) or Mn^{2+} at 2 mM (•). The ATP concentration added was 2 mM.

μmole/min/mg) when Mn^{2+} at either 1 or 2 mM was also added. It can be seen that Mn^{2+} is a more potent activator of the system but it has a strongly inhibitory effect when present in excess, unlike Mg^{2+}. This result also parallels one obtained with intact mitochondria.

That Mn^{2+} can protect against inhibition of the enzyme by Ca^{2+} is shown in fig. 3, which compares the reduction of activity as the Ca^{2+} concentration is raised in presence of either 8 mM Mg^{2+} or 2 mM Mn^{2+}. A similar difference was seen in another pair of Ca titrations made with ATP at 4 mM instead of 2 mM.

4. Discussion

It has long been known that Mn^{2+}, especially in presence of AMP, brings about shrinkage of swollen mitochondria [19]. This effect may be related to its protective effect against damage by Ca^{2+} [20]. Ca^{2+} might have been tranferred from cytoplasm to mitochondria during homogenisation, because the early preparations were not made with chelating agents in the homogenisation medium. A basis for the protective action, as suggested previously [10] is that Mn^{2+} counters the inhibitory effect of Ca^{2+} on pyruvate carboxylase described by Kimmich and Rasmussen [16], so that oxalacetate, and hence citrate, can be formed in response to the divalent cations. Besides acting as chelating agent, the citrate anion will balance more cations per osmole than will mono- and dicarboxylic acids. Since mitochondria behave in many respects as anion exchangers [6,11], the internal osmoles will be diminished.

An important but elusive aspect of the effects of intramitochondrial cations on enzyme activity in the intact particles is the question of what concentrations of free ions prevail in the face of chelation by ATP, citrate and, to lesser extents, by other compounds. Comparison between the effects on the isolated enzyme and in the mitochondria, indicates that even 25 μmole Mn^{2+}/g of protein in absence of added external Mg, does not maximally stimulate carboxylation, whereas this same content, along with the free Mg^{2+} in equilibrium with 0.67 mM externally, gives the same activation as is associated with the presence of 72 μmole Mm^{2+}/g protein without the added Mg^{2+} The low activity without Mn^{2+}, but with external Mg present, would not rule out the presence of free internal Mg at a concentration at 0.4 mM (as estimated from the citrate/isocitrate ratio [7]), and this could serve to supplement the free Mn^{2+} associated with an

internal content of 25 μmole/g to increase the enzyme activity (compare fig. 1, curves A and C). Since Mn^{2+} chelates are more associated that their Mg^{2+} analogues, it follows that, as metal ion is added, they give a sharper transition from a low concentration of free ion to a high concentration (analogous to the lesser buffering power of salts as their acid has a lower K_a). This difference in pattern between Mg^{2+} and Mn^{2+} presumably accounts for the sharp maximum in the concentration/activation curve found with the isolated enzyme, which acts as its own chelating agent.

It is worth speculating whether Mn may not have an anti-diabetogenic action [21] in that promotion of pyruvate carboxylation will tend to consume a source of acetyl CoA (the pyruvate) and then acetyl CoA itself in the condensation of oxalacetate to citrate.

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