Calcium Ion-Induced Uptakes and Transformations of Substrates in Liver Mitochondria

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Substrate-depleted rat liver mitochondria will reaccumulate malate, succinate, oxoglutarate, β -hydroxybutyrate and glutamate if provided with an energy source and Ca^{2+} (or Ca^{2+} and Mn^{2+}). The energy requirement for ion uptake by fresh mitochondria causes a transient oxidation of their NADH and presumably this leads to an increased oxaloacetate concentration. A consequence is the promotion of formation of citrate, which tends to remain in the particles, provided the pH is above 7. Analyses made of systems blocked with fluorocitrate show that citrate accumulates when Ca^{2+} is added with the following substrates; (a) pyruvate in the presence of ATP or malate, (b) palmitoyl-L(-)-carnitine in presence of malate and (c) oxoglutarate. Lowering the pH, even to 6.8, causes the citrate to emerge. This could be the basis of a cellular control mechanism. The generation of citrate in response to Ca^{2+} can explain the stoichiometry of one proton ejected per Ca^{2+} ion taken up. The new carboxyl group formed from acetyl-CoA when it condenses with oxaloacetate provides an internal anionic charge and a proton to emerge when Ca^{2+} enters.

The net gain of K^+ and Ca^{2+} by mitochondria is an energy-linked process (for K^+ see, e.g. Judah, McLean & Ahmed, 1965; for Ca^{2+} , see Judah & Ahmed, 1965; Chance, 1965). Along with a net gain of cations there is in general a less-than-equivalent loss of protons; electroneutrality is maintained by anion uptake. When there is a massive uptake of K^+ , a considerable quantity of anions also moves (Lynn & Brown, 1966; Harris, 1969), although there is also a variable amount of ejection of protons (Harris, Cockrell & Pressman, 1966; Rossi, Scarpa & Azzone, 1967). Ca^{2+} uptake, which requires energy but no special inducing agent, has been noted to be accompanied by uptake of phosphate (Lehninger, Rossi & Greenawalt, 1963), acetate (Rasmussen, Chance & Ogata, 1965), malate (Quagliariello & Palmieri, 1969), glutamate or hydroxybutyrate (Kimmich & Rasmussen, 1968).

The energy demand imposed by cation uptake leads to oxidation of endogenous nicotinamide nucleotides (Chance, 1965) and consumption of ATP. These changes lead to alteration of the internal metabolite pattern because various equilibria are shifted and enzyme activation and inhibition are altered. For example, Williamson, Coles, Olson & Herczeg (1968) showed that malate was converted into citrate when a load was imposed by inducing a high K^+ permeability with valinomycin.

It is, however, of more direct physiological importance to find the response to Ca^{2+} .

The priming of substrate uptake by Ca^{2+} is to be inferred from the observation by Haslam & Griffiths (1968) that oxaloacetate shows an energy-dependent reaction with endogenous NADH only if either the mitochondrial preparation has been made with 0.1 mM-EDTA in the homogenization step, or 50 μM - Ca^{2+} was added to the preparation made with 1 mM-EDTA* in the initial step. In the following account, measurements of substrates accompanying Ca^{2+} , and some substrate conversions brought about by Ca^{2+} , are described.

A preliminary account of some of this work has been published (Harris & Berent, 1969).

METHODS

The experiments were done with rat liver mitochondria prepared by using 0.25 M-sucrose containing 1 mM-EGTA and 0.05% of bovine serum albumin for homogenization. The first wash by resuspension and sedimentation was in 0.16 M-sucrose containing 35 mM-KCl, 1 mM-EGTA and 0.05% of bovine serum albumin; the second wash was in 0.16 M-sucrose containing 35 mM-KCl and final resuspension was in 0.25 M-sucrose. This procedure gives high yields with little or no 'fluffy' material; respiratory control is

* Abbreviations: EGTA, ethylenedioxybis(ethylamino)-tetra-acetic acid; TMPD, *NNN'*-tetramethylphenylene-diamine.

maintained by the cold (0°) storage suspension for 24 hr. The anion uptakes and effects of various additives on them were measured by withdrawing samples (0.2 ml.) from incubations at 20° with about 8 mg. of protein/ml. of a medium containing 120 mM-KCl and 20 mM-tris-chloride buffer, pH 7.4 (unless stated otherwise). The samples were layered on to silicone (General Electric Versilube F 50) itself layered on 1.5 M-HClO₄ in prepared plastic centrifuge tubes. As each sample was collected into a tube it was promptly spun in a Coleman Microfuge to separate the protein, which collected as a pellet at the bottom after passing successively through the silicone and the HClO₄. The fluid above the silicone was then withdrawn and acidified with HClO₄ to precipitate soluble protein. Samples of the acid extracts of the pellets were used for analysis after suitable neutralization. Samples of the treated supernatant were also analysed. Trace quantities of ³H₂O and [¹⁴C]sucrose were included in the medium so as to obtain measures of total water and sucrose-accessible water in the material that passed into the acid. Measurements with [¹⁴C]dextran showed that about 2.5 ml. of extraparticulate water/g. of protein was carried down, but the sucrose-accessible water (3–3.5 ml./g. of protein), which includes the water in the dextran-penetrated space, is the most useful value. It is assumed that all the smaller solutes are carried in the sucrose-accessible space at the same concentration as they have in the medium.

The quantities of substrate in the extract of the pellet and the concentrations in the supernatant were measured either by using ¹⁴C-labelled substances when their meta-

bolic conversion could be blocked, or by specific enzyme assays when metabolic changes could have occurred. Many experiments were concerned with the extent of conversion of one or two substrates into a third, and then multiple assays were made. Two or three similar samples were assayed at each stage in each experiment since there was no obvious time-dependence. Mean values are reported.

Assays for oxoglutarate, malate, pyruvate, phosphoenolpyruvate and citrate were made on neutralized portions of the acid extract of the protein recovered from beneath the silicone layer. Assays for ATP were made on portions of the incubation mixture sampled into cold HClO₄ and subsequently neutralized. Assays for NADH and NADPH were made on alkaline extracts obtained by sampling the suspension into ethanolic KOH followed by pH adjustment with triethanolamine hydrochloride. The assay methods were much as described by Bergmeyer (1963), adapted to the fluorimetric scale (see Maitra & Estabrook, 1964). Citrate was assayed by using citrate lyase and malate dehydrogenase in the presence of excess of NADH. Calcium analyses in the acid extract and of the supernatant fluid from the silicone separation were made with a Techtron atomic-absorption spectrophotometer.

Where depletion of energy-yielding compounds (and Ca²⁺ too) was required, the mitochondria were exposed to 1.5 μM-tetrachlorotrifluoromethylbenzimidazole and 0.5 mM-sodium arsenite for 10 min. at 20°. Then bovine serum albumin at 2 mg./ml. was added with excess of sucrose or KCl soln. and the mitochondria were sedimented and resuspended in sucrose or KCl.

Table 1. *Dependence on Ca²⁺ or Ca²⁺ + Mn²⁺ of the reaccumulation of some substrates by energy-depleted mitochondria in presence of added energy sources*

The medium contained 120 mM-KCl, 20 mM-tris-chloride buffer, pH 7.4, and in experiments nos. 1, 3, 5 and 6, 1 mM-tris-ascorbate. Additions were made sequentially. The values for contents were corrected for the amount of substrate carried down in the sucrose-accessible water of the pellet. They may include some adsorbed material.

Expt. no.	Anion being measured and concentration added	Addition	Mitochondrial content of the anion (μmoles/g. of protein)
1	Malate (0.6 mM)	Rotenone (2 μg./ml.)	1.4
		TMPD (20 μM)	1.7
		Ca ²⁺ (7 μmoles/g. of protein)	4.5
2	Malate (0.4 mM)	Rotenone (2 μg./ml.)	2.5
		Succinate (1 mM)	3.4
		Ca ²⁺ (16 μmoles/g. of protein)	5.1
3	Succinate (0.7 mM)	Antimycin (2 μg./ml.)	3.3
		TMPD (20 μM)	3.8
		Ca ²⁺ (7 μmoles/g. of protein)	5.1
4	β-Hydroxybutyrate (0.6 mM)	Rotenone (2 μg./ml.)	5.8
		Succinate (1 mM)	5.8
		Ca ²⁺ and Mn ²⁺ (8 μmoles of each/g. of protein) Plus 10 μmoles of each/g. of protein	7.4 10.8
5	Glutamate (0.8 mM)	Rotenone (2 μg./ml.)	2.3
		TMPD (20 μM)	2.6
		Ca ²⁺ and Mn ²⁺ (3.5 μmoles of each/g. of protein) Plus 5.3 μmoles of each/g. of protein	3.5 4.0
6	α-Oxoglutarate (1.3 mM)	Rotenone (2 μg./ml.)	3.4
		TMPD (20 μM)	3.4
		Ca ²⁺ and Mn ²⁺ (3.5 μmoles of each/g. of protein) Plus 5.3 μmoles of each/g. of protein	6.4 8.2

RESULTS

To relate net gain of substrate to that of Ca^{2+} it is preferable to start with mitochondria that have been depleted (see the Methods section). Untreated rat liver mitochondria carry pools of many anions (Bellamy, 1961) and these readily exchange for others without obvious cation or energy requirement (Harris & Manger, 1968, 1969). Depleted mitochondria reaccumulate citrate when given energy in the presence of dicarboxylic acid (Harris, 1968); other anions applied, even when given energy (from TMPD and ascorbate), are accumulated to a lesser extent, until an addition of Ca^{2+} or $\text{Ca}^{2+} + \text{Mn}^{2+}$ is made (Table 1). Some uptake on energizing could be due to the presence of Ca^{2+} that has leaked during the depletion process. Both the anion and Ca^{2+} are taken up. When present, the Mn^{2+} is presumed also to be taken up, because

in the presence of Ca^{2+} it is rapidly accumulated (Chance & Mela, 1968). Anions whose uptake was found to be increased in the presence of Ca^{2+} were glutamate, oxoglutarate, malate, succinate and β -hydroxybutyrate.

Special attention was paid to citrate because it is so much accumulated within the mitochondria (Harris, 1968, 1969). With undepleted mitochondria, which carry some endogenous malate, there was an increase in citrate content when Ca^{2+} was added to the system (Table 2a). With depleted mitochondria the provision of energy, and of Ca^{2+} , only slightly increased the citrate uptake (Table 2b), whereas further addition of malate led to considerable uptake. That is, for entry of citrate, malate is still necessary, although the extent of accumulation depends on the amount of Ca^{2+} supplied. The last point could be made more directly by adding Ca^{2+} and citrate together to

Table 2. *Dependence of mitochondrial citrate content on Ca^{2+} and other factors when citrate is supplied in the medium*

The medium contained 120 mM-KCl, 20 mM-tris-chloride buffer, pH 7.4, and tris-citrate with a trace of [^{14}C] citrate as specified. N.M., not measured. (a) With fresh mitochondria: the external citrate concentration was 0.7 mM. No respiratory inhibitor was added.

Ca^{2+} added ($\mu\text{moles/g.}$ of protein)	Mitochondrial Ca^{2+} ($\mu\text{moles/g.}$ of protein)	Mitochondrial citrate and products ($\mu\text{moles/g.}$ of protein)
None	17	23.2
3	N.M.	30.0
+ 4 = 7	N.M.	34.6
+ 5 = 12	32	39.1

(b) With depleted mitochondria: the external citrate concentration was 0.5 mM. Rotenone ($2 \mu\text{g./ml.}$) was present to stop oxidative metabolism of citrate.

Ca^{2+} or substance added	Mitochondrial Ca^{2+} ($\mu\text{moles/g.}$ of protein)	Mitochondrial citrate ($\mu\text{moles/g.}$ of protein)
None	4.5	1.2
Ca^{2+} (4 $\mu\text{moles/g.}$ of protein)	5.3	1.2
Ca^{2+} (to 10 $\mu\text{moles/g.}$ of protein)	5.8	1.6
TMPD (40 μM)	7.8	2.7
Malate (2 mM)	N.M.	7.5

Table 3. *Uptakes of Ca^{2+} and citrate and expulsion of pyruvate when equimolar calcium-citrate complex is added to a suspension of rat liver mitochondria in the presence of pyruvate (0.6 mM)*

The medium contained 120 mM-KCl, 20 mM-tris-chloride buffer, pH 7.4, and 0.6 mM-tris-pyruvate. Measurements were made by enzymic assay.

Calcium-citrate complex added ($\mu\text{moles/g.}$ of protein)	Mitochondrial Ca^{2+} ($\mu\text{moles/g.}$ of protein)	Mitochondrial pyruvate ($\mu\text{moles/g.}$ of protein)	Mitochondrial citrate ($\mu\text{moles/g.}$ of protein)	Concn. of citrate in the medium (μM)
None	8	14.8	1.4	48
13	18	6.7	4.2	76
+ 18 = 31	35	6.1	8.3	135
+ 36 = 67	56	1.8	23.5	256

undepleted mitochondria. The equimolar calcium-citrate complex remained in solution, even at 50mM, for some minutes after preparation. Successive additions of the complex to a suspension of mitochondria led to accumulation of citrate and uptake of the Ca^{2+} (Table 3).

These results show that Ca^{2+} , like K^{+} in the presence of valinomycin, acts as a counterion to neutralize entering anions. The following sections are concerned with consequences of energy consumption for movement of the Ca^{2+} (and Mn^{2+} when present).

Fig. 1. Oxidation of mitochondrial NADH in response to additions of Ca^{2+} ($\uparrow$). Samples were withdrawn for enzymic analysis before the addition of Ca^{2+} (as CaCl_2), at the fluorescence minimum just on addition, and during the subsequent recovery. The medium contained 0.25M-sucrose, 20mM-tris-chloride buffer, pH 7.2, 1.5mM-tris-malate and 1.5mM-tris-glutamate. The protein concentration was 4.6mg./ml. Each Ca^{2+} addition was $30\mu\text{moles/g. of protein}$.

Fig. 2. Transient oxidation of nicotinamide nucleotide shown by decrease of fluorescence (excitation at 360nm., measurement at 420nm.) in response to additions of different Ca^{2+} compounds or $\text{Ca}^{2+} + \text{Mn}^{2+}$. The maximum change in trace (a) is equal in magnitude to that obtained by the addition of $4\mu\text{moles}$ of tetrachlorotrifluoromethylbenzimidazole. Ca^{2+} ($44\mu\text{moles/g. of protein}$) was added as CaCl_2 in (a), as equimolar calcium-citrate complex in (b), as equimolar Ca^{2+} -EGTA complex in (c), and for (d) $22\mu\text{moles}$ of Ca^{2+} and $22\mu\text{moles}$ of $\text{Mn}^{2+}/\text{g. of protein}$ were added as CaCl_2 and MnCl_2 . At the time of mixing in the calcium salt there is an artifact; this has been omitted. The medium contained 120mM-KCl, 20mM-tris-chloride buffer, pH 7.4, 1.5mM-tris-glutamate and 1.5mM-tris-malate. The protein concentration was 5mg./ml.

Changes in endogenous nucleotides in response to Ca^{2+} uptake. It is well known that small additions of Ca^{2+} lead to transient oxidation of mitochondrial nicotinamide nucleotides, which can be succeeded by a lasting oxidation if the total quantity exceeds about $50\mu\text{moles/g. of protein}$. However, it is uncertain whether the two nicotinamide nucleotides of rat liver mitochondria are both involved in the changes. Fluorimetry is a convenient way to monitor the effect; assays for NADH and NADPH made on samples withdrawn and quickly stopped by alkaline extractions show that only the NADH content in fact changes (Fig. 1).

It is noteworthy that the fluorescence response to addition of the calcium-citrate complex is the same as that to an equal amount of calcium chloride, although the recovery is slower and does not show the increased final fluorescence given by the latter (Figs. 2a and 2b). Simultaneous addition of Mn^{2+} slows the response (Fig. 2d). Addition of Ca^{2+} as the EGTA complex causes a smaller but lasting oxidation (Fig. 2c). Enhanced fluorescence after addition of calcium chloride is not associated with a higher NAD(P)H concentration, so it must be due to some structural change. It should be noted that such structural change is prevented if either the calcium-citrate complex or Ca^{2+} with Mn^{2+} is added.

In similar experiments, the changes in the endogenous ATP content in response to additions of Ca^{2+} and P_i were followed by using the fluorescence as monitor and withdrawing samples for analysis. Without added P_i , the ATP concentration falls in response to added Ca^{2+} but partially recovers (Fig. 3); a second addition of Ca^{2+} produces a lasting depression of endogenous ATP content.

If now an amount of P_i about equivalent to the Ca^{2+} is added, there is a prompt rephosphorylation of the endogenous adenine nucleotide. Evidently phosphorylation had been inhibited by lack of endogenous P_i , which had been taken up by the Ca^{2+} .

These results show that the energy load imposed

by Ca^{2+} causes both a change in redox-state oxidation and a fall in ATP concentration. The next question is: what are the consequential changes in the metabolite pools?

Substrate transformations. Since oxidation of NADH favours oxaloacetate formation, there is a tendency for citrate to appear from condensation of the oxaloacetate with acetyl-CoA. The addition of Ca^{2+} to suspensions in the presence of pyruvate produces citrate; to avoid metabolic utilization of the latter, fluorocitrate was used to inhibit the aconitase reactions. Table 4 shows that with pyruvate as added substrate it is possible to accumulate citrate as it is formed from its precursors. In this series, and in contrast with the results in Table 3, there is little or no change in mitochondrial pyruvate concentration as the citrate content rises. This is presumably because, as Ca^{2+} is taken up, it provides more charges to neutralize anions and the pyruvate/citrate concentration ratio in the medium is much higher than in the conditions of Table 3.

If additions of Ca^{2+} are made without added ATP, the increment in mitochondrial citrate content is less, but if malate is also added there is an increase in citrate content, and the ability to gain Ca^{2+} seems also to depend on the presence of malate (Table 4b). Ca^{2+} given as the complex with EGTA also promotes citrate accumulation and is taken up. For example, the citrate content rose from (initial) 5.1 to 6.8 and then to 9.2 $\mu\text{moles/g.}$ with mitochondrial Ca^{2+} content rising from 11.5 to 21 and 25.5 $\mu\text{moles/g.}$ when the Ca^{2+} -EGTA complex was added.

Attention is drawn to the high concentration ratio of internal citrate to external citrate that is commonly observed. It is assumed that the citrate

Fig. 3. Consumption of mitochondrial ATP in response to additions of Ca^{2+} and restoration by the addition of P_i . Samples were withdrawn for enzymic analysis before the addition of Ca^{2+} , at the fluorescence minimum just on addition and during the subsequent recovery of fluorescence. The medium was that described in Fig. 1. The protein concentration was 5 mg./ml. The Ca^{2+} additions were 27 and 35 $\mu\text{moles/g.}$ of protein respectively, and P_i additions 54 $\mu\text{moles/g.}$ of protein.

Table 4. Formation and accumulation of citrate in response to Ca^{2+}

The medium contained 120 mm-KCl, 20 mm-tris-chloride buffer, pH 7.4, 10 μM -fluorocitrate and other additions as noted. N.M., not measured.						
(a) With pyruvate added at 0.9 mm and ATP at 0.5 mm.						
	Pyruvate		Citrate		Ca^{2+}	
Ca^{2+} added ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)
None	0.9	4.2	33	5.4	72	5.5
12	0.9	8.0	43	6.6	120	12
+ 25 = 37	N.M.	11.8	50	10.8	212	16
(b) With pyruvate added at 2.2 mm.						
	Pyruvate		Citrate		Ca^{2+}	
Addition	medium (mm)	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)
None	2.2	9.9	14	3.1	70	3
+ Ca^{2+} (11 $\mu\text{moles/g.}$)	2.2	9.3	8	3.0	N.M.	4
+ malate to 2 mm	N.M.	8.0	16	5.2	100	15

Table 5. *Formation and accumulation of citrate and pyruvate from malate and palmitoyl-L(-)-carnitine in response to additions of Ca²⁺ + Mn²⁺*

The medium contained 120 mM-KCl, 20 mM-tris-chloride buffer, pH 7.4 and 1 mM-tris-malate. The protein concentration was 7.2 mg./ml. in Expt. 1 and 8.8 mg./ml. in Expt. 2.

Additions in sequence	Pyruvate				Citrate			
	Expt. 1		Expt. 2		Expt. 1		Expt. 2	
	Mito- chondrial (μ moles/g.)	Medium (μ M)	Mito- chondrial (μ moles/g.)	Medium (μ M)	Mito- chondrial (μ moles/g.)	Medium (μ M)	Mito- chondrial (μ moles/g.)	Medium (μ M)
None	2.5	58	2.8	66	0.7	16	0.6	17
Palmitoyl-L(-)-carnitine to 16 μ M	2.5	94	1.7	66	1.0	27	1.3	10
Ca ²⁺ + Mn ²⁺ (12 μ moles of each/g.)	4.0	74	5.4	34	1.6	37	4.6	9
Palmitoyl-L(-)-carnitine to 30 μ M	4.6	74	5.5	66	2.7	100	6.8	14
Additional Ca ²⁺ + Mn ²⁺ (16 μ moles of each/g.)	4.1	65	4.6	67	5.2	55	6.4	11

content is dissolved in between 0.7 and 1 ml. of water/g. of protein (see Harris & van Dam, 1968; Pfaff, Klingenberg, Ritt & Vogell, 1968), so concentration ratios exceeding 100 are produced.

An alternative source of acetyl-CoA to participate in citrate condensation is fatty acid oxidation. To test the efficiency of citrate production from this source, palmitoyl-L(-)-carnitine was used in the presence of malate, the latter being the source of oxaloacetate. The carnitine derivative was chosen because it is supposed to be the form in which higher fatty acids are transferred across the membrane. The consequence of adding Ca²⁺ + Mn²⁺ to the system was an increase in mitochondrial citrate content (Table 5). Also, it was found that pyruvate was being formed, most of which remained in the mitochondria. Davis & Gibson (1969) have also reported an increasing pyruvate concentration with oxoglutarate as added substrate. They regard it as being due to decarboxylation of oxaloacetate.

The oxidation of oxoglutarate is of special interest in the context of energy loading, because this compound yields both GTP, from the substrate-level-phosphorylation step, and succinate, which in turn can be transformed into malate and oxaloacetate. The latter in the presence of GTP yields phosphoenolpyruvate in mitochondria of different species when high respiration has been induced, either with dinitrophenol (Scholte & Tager, 1965) or with oleate (Davis & Gibson, 1969). It was decided to test whether Ca²⁺ uptake would also bring about phosphoenolpyruvate formation. The mitochondria and medium were also assayed for pyruvate and citrate. It was found that in the presence of oligomycin there was appreciable formation of phosphoenolpyruvate (see Table 6). Presumably oligomycin preserves the endogenous

ATP to participate in pyruvate carboxylation. Alternatively, added ATP maintains the concentration of endogenous ATP. Important increases in citrate content were obtained in each condition, and also (not in Table 6) if ATP + oligomycin were both added. There was a comparatively constant ambient concentration of pyruvate, presumably carried with the preparation. The endogenous malate content was not measured in these experiments, but in others it was found to be 1–3.5 μ -moles/g., and was not diminished by citrate accumulation. If citrate is to be formed it is important that NADH should be oxidized. There is a comparatively small increase in citrate concentration if oleate is added, but if Ca²⁺ is then added there is a rise in citrate concentration from 4.5 to 7.8 μ moles/g.

pH and citrate retention. It is important to note that the retention of intramitochondrial citrate is pH-sensitive. If experiments like those described above were carried out at pH 7.2 or below there was little or no increase in mitochondrial citrate or Ca²⁺ content, but there was an increase in citrate concentration in the supernatant. This is consistent with there being a turnover of Ca²⁺, which imposes a load, but the combination of lower pH and energy load does not permit retention of the Ca²⁺ and citrate. Table 7 shows that the consequence of acidity is to discharge endogenous Ca²⁺ and citrate. Addition of Ca²⁺ now, in acid conditions, leads to the appearance of external citrate.

DISCUSSION

These results all illustrate the point that addition of Ca²⁺ to a mitochondrial suspension leads to citrate formation whether pyruvate, malate and

Table 6. *Appearance of citrate and phosphoenolpyruvate under the influence of Ca^{2+} added to a mitochondrial suspension in the presence of α -oxoglutarate*

The medium contained 120mm-KCl, 20mm-tris-chloride buffer, pH 7.4, 0.75mm- α -oxoglutarate and 20 μM -fluorocitrate. The protein concentration was 9.5mg./ml. in the experiment done in the presence of oligomycin (10 μg ./ml.), and 8mg./ml. in the experiment done in the presence of ATP (0.7mm). N.M., not measured.

Ca^{2+} added ($\mu\text{moles/g.}$ of protein)	Pyruvate				Phosphoenolpyruvate				Citrate			
	+ Oligomycin		+ ATP		+ Oligomycin		+ ATP		+ Oligomycin		+ ATP	
	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{mole/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)	mitochondrial ($\mu\text{moles/g.}$)	medium (μM)
None	7.6	116	5.5	59	1.0	29	0.5	80	3.0	72	2.6	51
15	7.0	103	5.3	53	2.3	72	0.5	N.M.	4.3	92	5.7	45
+ 13=28	6.0	94	4.9	25	2.7	67	0.9	77	8.9	67	6.2	34

fatty acid or oxoglutarate are the added substrates. The condensation of oxaloacetate with acetyl-CoA to form citrate provides an additional carboxyl group whose ionization yields a proton. If the active Ca^{2+} uptake drives the interior positive, this could account for the appearance of the proton outside the mitochondrion, leaving the interior with an ionized anion, corresponding to an extra hydroxyl group, as found by the titration method of Lehninger, Rossi, Bielawski & Gear (1967). The stoichiometry of 1 proton in the medium/ Ca^{2+} ion taken up commonly observed is then readily accounted for without assumption of improbable ionic species.

It may be questioned whether this explanation can hold when other substrates have been added to the mitochondrial suspension. As prepared rat liver mitochondria carry pyruvate at 5–8 $\mu\text{moles/g.}$ and malate at 1–4 $\mu\text{moles/g.}$, so some citrate precursors are always available, and, in absence of inhibitors, more can be generated from the tricarboxylic acid cycle operating on the added substrate on the one hand and from fatty acid oxidation on the other. The amount of Ca^{2+} that can be added without disrupting the mitochondrial membrane, so that the mitochondria will not all pass through the silicone during centrifugal separation, is limited to a final content of about 20 $\mu\text{moles/g.}$ without Mn^{2+} , and perhaps twice as much in the presence of equimolar Mn^{2+} , so it is important to start with mitochondria having a low Ca^{2+} content.

It may be noted that the conditions that have been described as favouring Ca^{2+} uptake without disruption of the mitochondria are also those that favour pyruvate carboxylase activity. This enzyme is necessary if pyruvate is to supply oxaloacetate for citrate condensation. Lindberg & Ernster (1954) noted that Mn^{2+} counters the disruptive effect of Ca^{2+} and it has been found in this laboratory (Miss J. Wimshurst, personal communication) that Mn^{2+} lessens the inhibition of pyruvate carboxylase by Ca^{2+} . Added ATP protects mitochondria against damage by Ca^{2+} and a supply of ATP is required for pyruvate carboxylation. As shown in Fig. 2, endogenous ATP is depleted by addition of Ca^{2+} so an exogenous supply is necessary. Oligomycin also protects against damage by Ca^{2+} and here presumably it prevents Ca^{2+} -induced adenosine triphosphatase activity from depleting the endogenous ATP, so leaving some available to promote the carboxylation process.

The shift of citrate from mitochondrion to medium in response to a fall of pH is perhaps important in the whole cell as a means to transfer citrate to regulate the cytoplasmic enzymes, notably phosphofructokinase (Garland, Randle & Newsholme, 1963) and acetyl-CoA carboxylase (e.g. Lowenstein, 1968). It implies that a move to

Table 7. *Discharge of mitochondrial citrate and Ca²⁺ by acid and the appearance of additional citrate in the medium in response to Ca²⁺ in acid conditions*

The medium contained 120mM-KCl, 20mM-tris-chloride buffer, initially pH 7.8, 0.8mM-tris-pyruvate and 16 μ M-fluorocitrate. HCl was used to adjust the pH.

pH	Citrate		Ca ²⁺	
	mitochondrial (μ moles/g. of protein)	medium (μ M)	mitochondrial (μ moles/g. of protein)	medium (μ M)
7.8	5.8	43	6.1	75
6.9	2.3	65	5.2	83
5.7	2.0	65	4.0	93
5.7*	2.0	88	4.0	232

* With an addition of Ca²⁺ + Mn²⁺.

acidity will inhibit glycolysis at the phosphofructokinase step and will promote the synthesis of fatty acid through the effect of citrate on the carboxylase.

It seems likely that Ca²⁺ is a component of a membrane carrier for anions perhaps analogous to the model given by the K⁺-valinomycin complex for cation transport. Anion permeation would then depend on retention of the carrier-bound Ca²⁺ as well as on a supply of energy to move the Ca²⁺-carrier complex. This would be consistent with the Ca²⁺ requirement found by Haslam & Griffiths (1968) and in the present paper.

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