The Dependence on Dicarboxylic Acids and Energy of Citrate Accumulation in Depleted Rat Liver Mitochondria

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The accumulation of some organic anions in the space inaccessible to sucrose of rat liver mitochondria was measured. In untreated mitochondria anions were apparently concentrated from 1 mM applied concentration by between five- and 22-fold, depending on their charge. After depletion of endogenous reserves either with uncoupling agent or with oligomycin uptakes were decreased. The accumulation of citrate was restored by combinations of a dicarboxylic acid (malate, succinate, maleate or *meso*-tartrate) and energy. The energy could either be provided by oxidation of a suitable dicarboxylic acid or from ascorbate in the presence of tetramethylphenylenediamine, or from ATP. The restoration of citrate uptake is not necessarily accompanied by a gain of K^+ , but a cation- and energy-linked citrate uptake can be induced with valinomycin. When citrate is added to mitochondria in the presence of malate the latter is competitively displaced. The anion accumulation could arise from an internal energy-linked positive potential.

Untreated mitochondria have the property of accumulating many different organic anions against a gradient even without an added energy supply. Gamble (1965) observed malate and citrate uptakes in the presence of cyanide as respiratory inhibitor. He also showed that citrate entry was accompanied by a displacement of organic and inorganic phosphates. Hence there appears to be a passive ion exchange. When the endogenous metabolites are consumed, whether by incubation with oxygen, or by addition of ADP and phosphate, or by exposure to an uncoupling agent, the ability of mitochondria to take up anions is impaired. These depletion procedures also decrease the K^+ content (see, e.g., Christie, Ahmed, McLean & Judah, 1965). It was shown (Harris, van Dam & Pressman, 1967) that respiratory inhibition resulting from excess of uncoupler is associated with K^+ loss and shrinkage; succinate contents after treatments leading to K^+ loss were lower than in the untreated mitochondria, so it was proposed that impaired substrate availability was the origin of the inhibition. The possibility of reversing the consequences of depletion has attracted attention.

Christie & Judah (1951) noted that oxidation of citrate could be inhibited by pretreatment with uncoupling agent or with glucose plus hexokinase, and they were able to restore the respiration by adding a crude NAD^+ preparation that contained some adenine nucleotide. Prolonged incubation of aged pigeon heart mitochondria with succinate eventually restores their capability to oxidize the substrate (Chance & Hagihara, 1960). Addition of

ATP restores the d'coumarol-stimulated rate of succinate oxidation by depleted mitochondria to the value characteristic of the fresh preparation (Azzone & Ernster, 1961).

Chappell (1964) found that the metabolism of isocitrate by depleted mitochondria required malate. Further metabolic studies (Williams, 1965; Ferguson & Williams, 1966; Chappell & Haarhoff, 1967) showed that other dicarboxylic acids would facilitate entry of the tricarboxylic acids, and Chappell & Crofts (1966), using mitochondrial swelling in the presence of ammonium salts of the acids as a criterion, obtained similar results, though their system also required phosphate. In none of these studies was accumulation of the tricarboxylic acid against a gradient either measured or necessary for the process being followed. An energy supply was not required. On the other hand Max & Purvis (1965), using a technique of centrifugal separation, found that energy was necessary if citrate was to be accumulated and remain during washing by resuspension of the mitochondria. The present study shows that a combination of a 'facilitating' dicarboxylic acid and energy is required to restore citrate accumulation in depleted mitochondria.

METHODS

Rat liver mitochondria were prepared and incubated as described by Harris & van Dam (1968) and Harris & Manger (1968). Samples (0.2 ml.) were taken from incubations having about 10 mg. of protein/ml. with substrate usually at 1 mM. The substrate included a proportion of ^{14}C -labelled compound, and H_2O was added to the medium.

Table 1. *Effect of depletion on uptakes of substrates applied at 1 mM to rat liver mitochondria at 20°*

Values were measured over 30 sec.–2 min. exposure and did not change with time. Uptakes are corrected for the content of a sucrose space assumed equal to the measured total water space minus 0.7 ml./g. of protein. Citrate, glutamate, hydroxybutyrate and malate were measured in the presence of rotenone (1 μ g./ml.), succinate in the presence of antimycin (1 μ g./ml.), and pyruvate and oxoglutarate in the presence of 0.5 mM-NaAsO₂. Two alternative media were used, containing either sucrose (250 mM), KCl (10 mM) and tris chloride, pH 7.4 (20 mM), or KCl (120 mM) and tris chloride, pH 7.4 (20 mM). No significant differences between the results were seen. Abbreviations: TTFB, tetrachlorotrifluoromethylbenzimidazole; BSA, bovine serum albumin.

Pretreatment	...	Corrected uptakes (μ moles/g. of protein)		
		None	<i>A</i>	<i>B</i>
			Oligomycin (1 μ g./mg.) for 5 min., rotenone or antimycin at time of anion addition	Shaken for 10 min. at 20° with TTFB (0.7 μ M) and NaAsO ₂ (1 mm), then 2.5 mg. of BSA added/ml.
Anion				
Citrate*		18.9, 15.8	6.3	1.3, 2.2, 0.2
L-Glutamate		5.5, 3.4	—	3.3, 2.6
DL- β -Hydroxybutyrate		5.8, 4.0	3.5	1.2
L-Malate		9.8, 10.9	4.4	0.4, 1.9
α -Oxoglutarate		9.8, 6.5	2.8	1.0
Pyruvate		4.1, 5.5	—	0.0
Succinate		12.5, 9.9	4.0	1.2

* Content includes about 10% of isocitrate.

RESULTS

The uptakes of different labelled anions from applied concentrations of 1 mM into untreated mitochondria are set out in Table 1 (under the column heading 'None'). They are the means of three values taken at intervals of 30 sec. There was no time-dependence between the first sample (at 30 sec. after mixing) and 10 min. in a control. These uptakes were all measured in the presence of respiratory inhibitors and appear to be passive exchanges. However, there was some endogenous ATP present, measurements of samples taken from the concentrated suspension at 0° gave values of 2–4 μ moles/g. of protein. The question was raised whether use of some of the endogenous ATP was involved in the anion exchange. If the mitochondria were mixed with the labelled substrate as before but with oligomycin present there was no obvious effect on the anion uptake. Since, however, oligomycin takes about 1 min. to inhibit phosphorylation, this result was inconclusive, for the anion uptake occurs rapidly. The anion uptake was decreased by adding oligomycin 5 min. before mixing with the labelled substrate and respiratory inhibitor (see column A in Table 1). This is as found by Harris *et al.* (1967). It is difficult to be sure that the result is due to removal of ATP as energy source, but observations made in a K⁺-free medium show that when oligomycin is added a K⁺ leakage sets in at a rate of 3–5 μ moles/g. of protein/min. The most effective way to decrease the anion uptakes was previously to expose the mitochondria

to an uncoupling agent. For this 0.7 μ M-tetrachlorotrifluoromethylbenzimidazole was used in the presence of 1 mM-arsenite. After 10 min. at 20° bovine serum albumin (2.5 mg./ml.) was added to combine with the uncoupler (Beechey, 1966). As shown in column B in Table 1, the net uptakes, corrected for contents of the sucrose-accessible space, are low; only glutamate is relatively unaffected (in agreement with a remark of Azzi, Chappell & Robinson, 1967).

In view of the extensive literature on the restoration of the metabolism of citrate by malate it was decided to investigate citrate accumulation. The net accumulation was measured in three conditions: (1) with untreated mitochondria; (2) with mitochondria depleted by exposure to uncoupler; (3) as (2), but with added dicarboxylic acid. To preclude oxidation of the citrate, all the experiments were made in the presence of rotenone and succinate was used as the dicarboxylic acid.

The results are shown in Fig. 1. The undepleted preparation had a maximum citrate capacity of 22 μ moles/g. of protein with K_m 1.0 mM (curve A). After depletion the citrate uptakes were not much more than the estimated contents of the sucrose space; the maximum capacity was 3 μ moles/g. of protein with K_m still 1.0 mM. Addition of succinate to another depleted preparation brought back the citrate capacity to 33 μ moles/g. of protein with K_m 1.0 mM.

The addition of malate or succinate to the depleted preparation rapidly restored citrate uptake, as shown in Fig. 2. If, however, malate was

Fig. 1. Double-reciprocal plots of concentration versus net citrate uptakes by untreated mitochondria (curves A), depleted mitochondria (curve B) and depleted mitochondria plus succinate (curves C). Curves A and C from (a) are transposed to the other scale of (b) for comparison with curve B. Sets of three samples from each concentration were taken from incubations at 20° in a medium containing sucrose (250 mM), tris chloride, pH 7.4 (20 mM), $[1,5\text{-}^{14}\text{C}_2]\text{citrate}$ and carrier to the concentration plotted, all with $1\mu\text{g.}$ of rotenone/ml. Mean contents of 'citrate' included about 10% of isocitrate. Deductions were applied for the citrate estimated to be present in the sucrose-accessible space. This space was assumed to be 0.7 ml./g. of protein less than the measured $^3\text{H}_2\text{O}$ space. The depletion was as given for column B in Table 1. The protein concentration was 10 mg./ml. Mitochondrial 'citrate' is the content of citrate, including isocitrate and aconitate, corrected for the citrate in the sucrose-accessible space, referred to the mitochondrial protein. $[\text{Citrate}]$ is the external concentration of citrate.

Fig. 2. Successive values of 'citrate' uptakes during incubations of depleted mitochondria with additions at arrows of either malate (1 mM) or succinate (1 mM) in the presence of rotenone ($1\mu\text{g./ml.}$). The medium was as in Fig. 1 with labelled citrate at 1 mM. Malate was added to the succinate-supplemented incubation at $6\frac{1}{2}\text{ min.}$ and may have caused some displacement of the accumulated citrate.

Fig. 3. Successive values of 'citrate' uptakes during incubation of depleted mitochondria with additions of malate (1 mM) in the presence of rotenone ($1\mu\text{g./ml.}$) and then of succinate (1 mM). The medium was as in Fig. 1, with labelled citrate at 1 mM.

added in the presence of rotenone, so that it was not oxidized, there was only a small citrate uptake (Fig. 3), whereas succinate, which could still be

oxidized, led to a considerable uptake. Other combinations of dicarboxylic acid and energy source were also effective, as summarized in Table 2.

Dicarboxylic acids that were not oxidized, or that had their oxidation inhibited, caused little uptake; energy supply from ascorbate oxidation (coupled through tetramethylphenylenediamine) or added ATP had little or no effect, whereas combinations of acid and energy supply led to gains of between 5 and $12\mu\text{moles/g.}$ of protein.

Table 2. Contents of citrate after incubation of mitochondria depleted by method B (Table 1) with citrate at 1 mM, before and after various additions

Dicarboxylic acids that can be oxidized bring about a net increase. Non-metabolized acids have relatively little effect until a source of energy is also provided. Tetramethylphenylenediamine (TMPD) was used in the presence of 3 mM-ascorbate.

Inhibitor	Content of citrate before addition (μ moles/g. of protein)	Addition 1	Content of citrate (μ moles/g. of protein)	Addition 2	Content of citrate (μ moles/g. of protein)
None	7.3	Malate (1 mM)			19.4
Rotenone	5.2	Succinate (1 mM)			16.8
Rotenone	4.9	Malate (1 mM)	7.2	Succinate (4 mM)	17.2
Rotenone	6.3	TMPD (30 μ M)	6.3	Malate (1.5 mM)	15.0
Rotenone	5.8	<i>meso</i> -Tartrate (1 mM)	9.5	TMPD (50 μ M)	17.4
Rotenone	5.2	TMPD (30 μ M)	5.6	<i>meso</i> -Tartrate (2.5 mM)	10.0
Rotenone	6.3	TMPD (30 μ M)	7.5	Maleate (2.5 mM)	12.9
Antimycin A	4.2	Succinate (2.5 mM)	7.5	TMPD (30 μ M)	10.6
Antimycin A	5.4	TMPD (20 μ M)	7.5	Acetylenedicarboxylic acid (2.5 mM)	10.4
Rotenone	3.1	ATP (0.7 mM)	3.8	Malate (1.3 mM)	14.0

Fig. 4. Competitive displacement of malate by citrate. Values of malate uptake by untreated mitochondria were measured at three different malate concentrations (O, 0.28 mM; ●, 0.57 mM; Δ, 1.32 mM). Successive additions of citrate were made and malate contents were measured after each. The malate uptakes were corrected for the amounts in the sucrose-accessible space before being plotted. The medium contained KCl (120 mM), tris chloride, pH 7.4 (20 mM), rotenone (1 μ g./ml.) and [14 C]malate as specified. Mitochondrial malate is the content of malate corrected for that in the sucrose-accessible space, referred to the mitochondrial protein.

If ADP was added to the system before citrate uptake had taken place the concentration of citrate subsequently attained was lower than that reached

Fig. 5. Uptake and release of 'citrate' (●) and K^+ (○) caused by additions of valinomycin (5 μ g./g. of protein) and nigericin (15 μ g./g. of protein) to a suspension of rat liver mitochondria. The medium contained citrate (1 mM), sucrose (250 mM), KCl (10 mM) and tris chloride, pH 7.4 (20 mM). The temperature was 25° and protein concentration was 10.5 mg./ml. The 'citrate' includes the labelled metabolic products from [$1,5-^{14}C_2$]citrate.

in controls containing the other components only. For example, in a pair of experiments the mixture containing ADP led to an uptake of 8 μ moles/g. of protein instead of 11 μ moles/g. of protein in the control.

Gamble (1965) showed that citrate uptake is accompanied by displacement of orthophosphate and organic phosphates. If the mitochondria had been exposed to malate then citrate addition displaced the malate (Fig. 4). The displacement can be described as a competitive adsorption, with citrate having K_i 1.4 mM. It is important to remark, however, that accumulation resulting from an internal positivity would prefer citrate to malate on

account of the former's higher charge. To maintain neutrality, malate would then be driven out.

The amounts of mitochondrial citrate accumulated in these experiments with 1mM external concentration were similar to those found by Max & Purvis (1965) after exposure to 20mM concentrations followed by washing. The time-dependence described by Max & Purvis (1965) may have arisen because of a slow binding making the adsorbed citrate able to resist washing rather than because of slowness of the actual entry.

The possibility that the energy requirement for restoration of the citrate uptake reflects an energy-dependent cation uptake, also considered and rejected by Max & Purvis (1965), could not be supported. Analyses for K^+ and Mg^{2+} failed to show changes linked with citrate gain. The energy-dependent citrate uptake could be elicited in K^+ -free media and in media to which EDTA had been added to chelate Mg^{2+} or Ca^{2+} .

An energy- and cation-linked citrate uptake could, however, be shown when K^+ uptake was induced by the addition of valinomycin (Fig. 5). Labelled material (citrate and its metabolic products) accumulated with the K^+ and were released when K^+ was released.

DISCUSSION

Three processes can lead to citrate uptake in isolated rat liver mitochondria given external citrate. Without prior depletion the citrate enters in exchange for endogenous anions in the absence of respiration or addition of ATP. For this to occur some endogenous reserve of malate and ATP presumably must be present. Under conditions in which endogenous ATP is expected to be depleted, namely with oligomycin present and with respiration blocked, the uptakes are decreased though not abolished. The same pattern holds for the other substrates investigated, with the possible exception of glutamate. It is possible that the endogenous ATP sustains an internal positivity that causes the accumulation. This explanation would require that the accumulation ratio should increase with the anion's charge.

More severe depletion, by which both ATP and substrates are presumably removed, largely removes

the ability to take up anions, including citrate. In this condition the second mode of citrate uptake is exposed; this has a requirement for the presence of a dicarboxylic acid as found by other workers, who also showed that mere entry of tricarboxylic acids, without there necessarily being any accumulation, did not require energy. The third process leading to citrate uptake is a massive net gain of K^+ such as valinomycin induces.

It will be of importance to investigate to what extent the uptakes of other substrates can be restored after they have been impaired by depletion. Preliminary experiments with succinate indicate that its uptake can be increased by ATP addition (E. J. Harris, unpublished work).

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