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Role of Calcium Ions in the Regulation of Intramitochondrial Metabolism

PROPERTIES OF THE Ca^{2+}-SENSITIVE DEHYDROGENASES WITHIN INTACT UNCOUPLED MITOCHONDRIA FROM THE WHITE AND BROWN ADIPOSE TISSUE OF THE RAT

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1. Increasing concentrations of both Ca^{2+} and Sr^{2+} (generated by using EGTA buffers) resulted in 4-fold increases in the initial activity of pyruvate dehydrogenase within intact uncoupled mitochondria from rat epididymal adipose tissue incubated in the presence of the ionophore A23187, ATP, Mg^{2+} and oligomycin. The $k_{0.5}$ values (concentrations required for half-maximal effects) for Ca^{2+} and Sr^{2+} were 0.54 and 7.1 μM respectively. In extracts of the mitochondria, pyruvate dehydrogenase phosphate phosphatase activity was stimulated about 4-fold by Ca^{2+} and Sr^{2+}, with $k_{0.5}$ values of 1.08 and 6.4 μM respectively. 2. NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase appeared to be rate-limiting in the oxidation of *threo*-D_s-isocitrate and oxoglutarate by uncoupled mitochondria from brown adipose tissue of cold-adapted rats. Ca^{2+} (and Sr^{2+}) diminished the K_m for the oxidation of both *threo*-D_s-isocitrate and oxoglutarate. The kinetic constants for these oxidations were very similar to those obtained for the activities of NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase in extracts of the mitochondria. In particular, the $k_{0.5}$ values for Ca^{2+} were all in the range 0.2–1.6 μM and Sr^{2+} was found to mimic Ca^{2+}, but with $k_{0.5}$ values about 10 times greater. 3. Overall, the results of this study demonstrate that the activities of pyruvate dehydrogenase, NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase may all be increased by Ca^{2+} and Sr^{2+} within intact mitochondria. In all cases the $k_{0.5}$ values are close to 1 and 10 μM respectively, as found for the separated enzymes. Experiments on brown-adipose-tissue mitochondria incubated in the presence of albumin suggest that it may be possible to use the sensitivity of the dehydrogenases to Ca^{2+} as a means of assessing the distribution of Ca^{2+} across the mitochondrial inner membrane.

Studies in our laboratory have identified three mammalian mitochondrial dehydrogenases which, in mitochondrial extracts or after purification, are activated by Ca^{2+}, with a $k_{0.5}$ (concentration required for half-maximum effect) for Ca^{2+} close to 1 μM. These enzymes are the pyruvate dehydrogenase complex, NAD^+–isocitrate dehydrogenase (EC 1.1.1.41) and the 2-oxoglutarate dehydrogenase complex. All are located exclusively within the mitochondrial inner membrane. The effects of Ca^{2+} on pyruvate dehydrogenase activity are brought about by changes in the proportion of the enzyme in its active non-phosphorylated form. Pyruvate dehydrogenase phosphate phosphatase is activated by Ca^{2+} (Denton *et al.*, 1972, 1975), whereas pyruvate dehydrogenase kinase, at least from heart muscle, may be inhibited by Ca^{2+} (Cooper *et al.*, 1974); thus the effect of Ca^{2+} is to increase the maximum activity of pyruvate dehydrogenase, with little or no effect on the K_m of the enzyme for its substrate, pyruvate. In contrast, the effects of Ca^{2+} on NAD^+–isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase appear to be more direct and lead to a greatly decreased K_m for their substrates (*threo*-D_s-isocitrate and oxoglutarate respectively) with little or no effect on the $V_{max.}$ (Denton *et al.*, 1978; McCormack & Denton, 1979). The enzymes appear to have very similar properties in extracts of all mammalian mitochondria so far studied, including those from pig heart and the following rat tissues: epididymal white adipose

Abbreviations used: Mops, 4-morpholinepropanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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tissue, interscapular brown adipose tissue, liver, kidney, heart, skeletal muscle and brain (Denton *et al.*, 1972, 1975, 1978, 1980; McCormack & Denton, 1979; the present paper; unpublished work by R. M. Denton, J. G. McCormack and 2nd-year students reading biochemistry at the University of Bristol).

Taken together, the above findings strongly suggest that Ca^{2+} may be an important regulator of oxidative metabolism in mammalian mitochondria. In particular, the possibility is raised that certain hormones and transmitters may bring about their effects on mitochondrial metabolism through altering the concentration of Ca^{2+} in mitochondria (Denton & Halestrap, 1979). However, we realize the need to be cautious when ascribing regulatory importance to enzyme properties that have only been observed with isolated enzymes (Denton *et al.*, 1978; McCormack & Denton, 1979). We have therefore sought more direct evidence, using intact mitochondria, for a regulatory role for intramitochondrial Ca^{2+}. In the present paper, we describe investigations designed to establish the sensitivities of the three dehydrogenases to Ca^{2+} when they are located within mitochondria. We have approached the problem by studying the effects of changes in extramitochondrial Ca^{2+} on pyruvate dehydrogenase activity and on oxidation of *threo*-D_s-isocitrate or oxoglutarate in uncoupled mitochondria. Under these conditions there will be little or no pH gradient (ΔpH) or membrane potential ($\Delta\Psi$) across the mitochondrial inner membrane, and thus it is reasonable to expect that the concentration of Ca^{2+} in the mitochondrial matrix will be close to that outside the mitochondria. The studies on oxidation of *threo*-D_s-isocitrate and oxoglutarate have been carried out on rat brown-adipose mitochondria, which have the advantage of being easily prepared in an uncoupled state without the need to add exogenous uncouplers (Nicholls, 1979). Moreover, the activities of NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase appear to be rate-limiting in the oxidation of their respective substrates. In the studies reported in the following paper (Denton *et al.*, 1980), we have explored the effects of changing the extramitochondrial Ca^{2+} in the 'physiological' range on the activity of oxoglutarate dehydrogenase and pyruvate dehydrogenase in coupled rat heart mitochondria and demonstrate marked activation of both enzymes in this range.

Experimental

Chemicals and biochemicals

FCCP, oligomycin, all coenzymes and substrates (except isocitrate) were from Boehringer Corp. (London) Ltd., Lewes, East Sussex BN7 1LG, U.K. DL-Isocitrate [trisodium salt, containing 50% (w/w) *threo*-D_s-isocitrate], EGTA, EDTA and other chemicals were the highest grade available from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. The ionophore A23187 was kindly given by Dr. R. L. Hamill, Eli Lilly and Co. Indianapolis, IN, U.S.A., Rotenone was from Aldrich Chemical Co., Gillingham, Dorset SP8 4BR, U.K. Preparation of *threo*-D_s-isocitrate (K^+ salt) was as described by Denton *et al.* (1978).

Pig heart pyruvate dehydrogenase (free of phosphatase activity) was prepared essentially as described by Cooper *et al.* (1974) and converted into pyruvate dehydrogenase [^{32}P]phosphate as described by Denton *et al.* (1972). The preparation had little or no pyruvate dehydrogenase activity, but, after treatment with pig heart pyruvate dehydrogenase phosphate phosphatase in the presence of 25mM-$MgCl_2$ and 1mM-$CaCl_2$, all ^{32}P was released and the original pyruvate dehydrogenase activity was regained. The extent of phosphorylation was approx. 0.3nmol of P_i/unit of original pyruvate dehydrogenase activity. Pyruvate dehydrogenase phosphate phosphatase was prepared from pig heart as described by Severson *et al.* (1974). Arylamine acetyltransferase was prepared as described by Coore *et al.* (1971).

Bovine serum albumin was obtained from Sigma (London) Chemical Co., Poole, Dorset BH16 7NH, U.K., and defatted by the method of Chen (1967).

Preparation of mitochondria

White-adipose-tissue mitochondria were prepared from the epididymal fat-pads of male rats (170–200g). The rats were housed at room temperature, fed *ad libitum* on a stock laboratory diet (Breeding Diet: Oxoid Ltd., Basingstoke, Hants., U.K.) and killed by decapitation. Groups of fat-pads (6–12) were incubated with shaking for 30min at 37°C in bicarbonate-buffered medium (4ml/g), gassed with O_2/CO_2 (19:1) (Krebs & Henseleit, 1932) and containing glucose (2mg/ml). The pads were lightly blotted and rapidly disrupted in 2ml of ice-cold sucrose extraction medium [250mM-sucrose, 20mM-Tris/HCl, 20mg of defatted albumin/ml, 7.5mM-glutathione (reduced), 2mM-EGTA, pH7.4]/pad in a Polytron PT-20 tissue homogenizer set at one-third full speed for 3.5s. The fat plug was separated by centrifugation at 1000*g* for 90s at 4°C and then the mitochondria were sedimented by centrifugation of the infranatant at 9000*g* for 5min at 4°C. The mitochondria were resuspended in 125mM-KCl/20mM-Tris/HCl (pH7.2) at 0°C (at about 20mg of protein/ml). All preparations of the mitochondria used in this study exhibited good respiratory control, with ratios 4–6 (Chance & Williams, 1956) for the oxidation of pyruvate (5mM) plus malate (0.5mM), 3–5 for the

oxidation of succinate (5mM) and 4–8 for the oxidation of oxoglutarate or *threo*-D_s-isocitrate (5mM) plus malate (0.5mM).

Brown-adipose-tissue mitochondria were prepared from the interscapular fat tissue of fed female rats (200–300g) which had been cold-adapted for 4–6 weeks at 5°C, by a slight modification of the procedure described above for white-adipose-tissue mitochondria. The rats were first anaesthetized with Sagatal (pentobarbital: 60mg/kg). Interscapular brown adipose tissue was removed and immediately disrupted in 4ml of the ice-cold sucrose medium/g in the Polytron PT-20. Preparation was then as described above. In some experiments the mitochondria were washed with sucrose-based extraction medium without albumin, and then resuspended; this had no appreciable affect on the properties of the mitochondria. The mitochondria oxidized a variety of substrates rapidly when incubated in KCl-based medium in the absence of ADP (see Table 2), including pyruvate, citrate, *threo*-D_s-isocitrate, oxoglutarate and palmitoylcarnitine (all in the presence of malate) or succinate alone. There was no stimulation of respiration by added ADP under these conditions. If both albumin (1mg/ml) and GDP (0.5mM) were added to the incubation medium, the rate of oxidation of all the substrates above was decreased to 20–40% of the original rate. The subsequent addition of ADP (2mM) increased this rate 1.5–2.5-fold. Similar properties have been described previously for mitochondria of brown adipose tissue from a number of sources (see Cannon & Lindberg, 1979; Nicholls, 1979).

Incubation of mitochondria

Mitochondria were incubated at 30°C in KCl-based medium (125mM-KCl, 20mM-Tris/HCl, pH 7.0–7.2) saturated with air and containing the additions indicated.

Extraction of mitochondria

Samples (0.5–5mg of protein) of mitochondria were sedimented by centrifugation for 60s at 10000*g* in an Eppendorf 3200 mini-centrifuge and the pellet of mitochondria was immediately frozen with liquid N_2. The mitochondria were then extracted by freezing and thawing three times in 500μl of 100mM-KH_2PO_4 buffer (pH 7) containing 1mM-dithiothreitol and 50μl of rat serum/ml (to prevent proteolysis: Wieland, 1975). For the assay of pyruvate dehydrogenase activity in white-adipose-tissue mitochondria, 2mM-EDTA was also added to this extraction medium to prevent the interconversion of the phosphorylated and non-phosphorylated forms of the enzyme (Coore *et al.*, 1971). For the assay of NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase activity in brown-adipose-tissue mitochondria, 1mM-ADP was present in this extraction medium and the extracts were centrifuged for 5min at 10000*g* before assay of enzyme activity.

Assay of enzyme activities

Pyruvate dehydrogenase was assayed with arylamine acetyltransferase as described by Coore *et al.* (1971). Total pyruvate dehydrogenase activity was taken as that present after incubation of extracts with pig heart pyruvate dehydrogenase phosphate phosphatase (0.5unit/ml), 25mM-$MgCl_2$ and 1mM-$CaCl_2$ for 15min at 30°C.

Pyruvate dehydrogenase phosphate phosphatase was assayed as the release of $[^{32}P]P_i$ from pig heart pyruvate dehydrogenase [^{32}P]phosphate (Denton *et al.*, 1972; Stansbie *et al.*, 1976). Final concentrations present in the assay were as follows: 20mM-potassium phosphate, pH 7.0, 25mM-$MgCl_2$, 1mM-dithiothreitol, pyruvate dehydrogenase [^{32}P]-phosphate (1nmol of protein-bound phosphate/ml), 5mM-EGTA and appropriate additions of $CaCl_2$ or $SrCl_2$.

NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase activities were assayed at 30°C by following the production of NADH at 340nm as described by Denton *et al.* (1978) and McCormack & Denton (1979) respectively in 50mM-Mops buffer, pH 7.0–7.2, containing 2μg of rotenone/ml, 1mM-dithiothreitol and other additions as indicated.

Enzyme activities are expressed as units of enzyme activity where (except for pyruvate dehydrogenase phosphate phosphatase) a unit of activity is taken as that amount of enzyme which transforms 1μmol of substrate/min at 30°C. For pyruvate dehydrogenase phosphate phosphatase, units are calculated in terms of μmol of P_i released/min.

Assay of total protein

Protein was determined on samples of mitochondria by a modified biuret method (Gornall *et al.*, 1949), standardized with bovine serum albumin.

Assay of mitochondrial ATP content

A sample (100μl) of mitochondria and medium was removed at the indicated time and mixed vigorously with an equal volume of 5% (w/v) $HClO_4$ with a vortex mixer. After centrifugation at 16000*g* for 2min, ATP was assayed in a sample of supernatant by the method of Stanley & Williams (1969).

Measurement of oxygen uptake

Rates of respiration of mitochondria were recorded using a small Clark-type oxygen electrode (constructed in the Medical School Glass Workshop, University of Bristol, by Mr. Malcolm Fowler).

Mitochondria (0.25–1 mg of protein) were incubated in a total volume of 1.0–1.2 ml at 30°C in KCl-based medium saturated with air and containing the additions indicated.

Handling of data

The use of EGTA buffers and calculation of free and bound concentrations of bivalent metal ions were as described by Denton *et al.* (1978) and McCormack & Denton (1979), with the necessary compensations for changes in pH. Because of the sensitivity of EGTA–Ca buffers and also the activity of both NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase to small changes in pH, particular care was taken in these studies to ensure that buffers were at the required pH at 30°C.

Kinetic constants were calculated by fitting data to the equations of the type

$$v = V_{max.}/\{1 + (K_m/[S])^n\}$$

by using a non-linear least-squares regression program written for a Hewlett–Packard 8945 computer by Dr. Paul England of this Department.

Results and Discussion

Effects of Ca^{2+} and Sr^{2+} on the activity of pyruvate dehydrogenase phosphate phosphatase in extracts of white-adipose-tissue mitochondria

In the presence of a saturating concentration of Mg^{2+}, Ca^{2+} stimulated about 4-fold the activity of pyruvate dehydrogenase phosphate phosphatase assayed in extracts of white-adipose-tissue mitochondria with pig heart pyruvate dehydrogenase [^{32}P]phosphate as substrate (Fig. 1*a*). The calculated $k_{0.5}$ (±S.E.M.) was 1.08 ± 0.07 μM (16 observations). This is in close agreement with previous estimates for extracts of mitochondria prepared from isolated rat epididymal fat-cells (Denton *et al.*, 1972; Severson *et al.*, 1974). Some preliminary indications that Sr^{2+} may mimic the effect of Ca^{2+} on activity of this phosphatase have been obtained previously (Severson *et al.*, 1974; Randle *et al.*, 1974), but no estimates of kinetic parameters have been reported. As shown in Fig. 1(*a*), Sr^{2+} will elicit a very similar maximum increase in phosphatase activity to that observed with Ca^{2+}, but the $k_{0.5}$ for Sr^{2+} is considerably greater than that for Ca^{2+}. The value (±S.E.M.) calculated from the data of Fig. 1(*a*) was 6.36 ± 0.84 μM (16 observations).

Effects of Ca^{2+} and Sr^{2+} on the activity of pyruvate dehydrogenase in uncoupled white-adipose-tissue mitochondria incubated with ATP and the ionophore A23187

Previous work has shown that depleting white-fat-cell mitochondria of Mg^{2+} and Ca^{2+} by incubation with the ionophore A23187 and EGTA leads to complete loss of pyruvate dehydrogenase phosphate

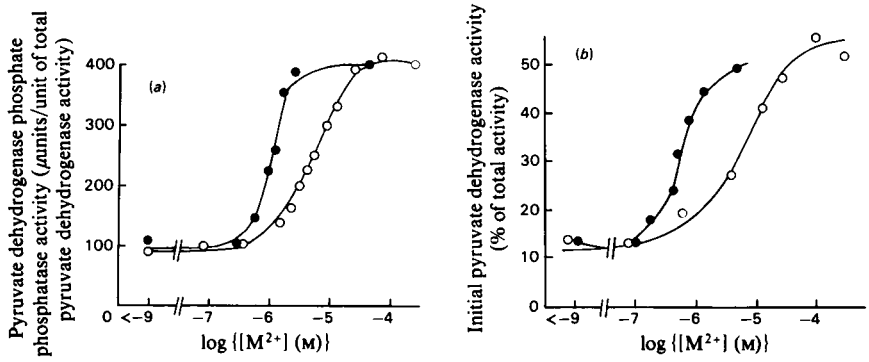

Fig. 1. *Effects of Ca^{2+}* (●) *and Sr^{2+}* (○) *on* (*a*) *the activity of pyruvate dehydrogenase phosphate phosphatase in extracts of white-adipose-tissue mitochondria and on* (*b*) *pyruvate dehydrogenase activity within intact white-adipose-tissue mitochondria incubated with uncoupler, the ionophore A23187 and exogenous ATP*

(*a*) Mitochondria from rat epididymal fat-pads were prepared and extracted as described in the Experimental section. Pyruvate dehydrogenase phosphate phosphatase was assayed in samples of the extracts as the release of $^{32}P_i$ from pig heart pyruvate dehydrogenase [^{32}P]phosphate at pH 7.0 and 30°C. Indicated concentrations of Ca^{2+} (●) and Sr^{2+} (○) were obtained by using 5 mM-EGTA buffers. Further details are given in the Experimental section. Results are the mean values taken from two to four separate experiments; calculated $k_{0.5}$ values are given in the text. (*b*) Mitochondria from rat epididymal fat-pads were incubated for 5 min at a concentration of 0.5 mg of protein/ml in 125 mM-KCl, 20 mM-Tris/HCl, 2 mM-potassium phosphate, pH 7.3, containing 0.5 μM-FCCP, ionophore A23187 (2.5 μg/ml), oligomycin (5 μg/ml), $MgCl_2$ (5 mM), and ATP (5 mM), together with 5 mM-EGTA buffers to yield the indicated concentrations of Ca^{2+} (●) or Sr^{2+} (○). After sedimentation by centrifugation (10000*g*-min), the mitochondria were immediately frozen in liquid N_2, extracts prepared and assayed for initial and total pyruvate dehydrogenase activity as described in the Experimental section. Results are the mean of values obtained in two separate experiments; calculated $k_{0.5}$ values are given in the text.

phosphatase activity; restoration of activity required addition of Mg^{2+} plus either Ca^{2+} or Sr^{2+} to the mitochondrial incubation medium (Severson *et al.*, 1974). These studies allowed the conclusion that the phosphatase when located within mitochondria was activated by Ca^{2+} or Sr^{2+}, but no real measure of the range of intramitochondrial Ca^{2+} or Sr^{2+} concentrations necessary to achieve activation was obtained.

We have now estimated the $k_{0.5}$ for Ca^{2+} and Sr^{2+} by incubating white-fat-cell mitochondria with $MgCl_2$, the uncoupler FCCP and the ionophore A23187 so that the intramitochondrial concentration of Ca^{2+} (or Sr^{2+}) should be very close to that in the mitochondrial incubation medium (Fig. 1*b*). It was also necessary to add ATP and oligomycin to ensure adequate pyruvate dehydrogenase kinase activity under these conditions. Appreciable disappearance of the added ATP occurred even in the presence of the high concentration of oligomycin used. In the experiments of Fig. 1(*b*), we assayed initial pyruvate dehydrogenase activity after incubation of the mitochondria for 5 min at 30°C with various concentrations of Ca^{2+} and Sr^{2+} generated by using EGTA buffers. Loss of ATP over the 5 min period varied between 7 and 15%, but no appreciable differences in the rate of decline of extramitochondrial ATP was observed with different concentrations of Ca^{2+} and Sr^{2+}. Very similar results to those shown in Fig. 1(*b*) were also obtained after incubation of mitochondria for 10 min at 30°C. As the concentration of extramitochondrial Ca^{2+} or Sr^{2+} was increased, the initial activity of pyruvate dehydrogenase rose (Fig. 1*b*); there was no change in the total activity of pyruvate dehydrogenase. The sensitivity to changes in extramitochondrial Ca^{2+} and Sr^{2+} concentrations was very similar to that found with pyruvate dehydrogenase phosphate phosphatase in mitochondrial extracts (Fig. 1*a*). Apparent $k_{0.5}$ values (±S.E.M.) calculated from the data of Fig. 1(*b*) for the increase in pyruvate dehydrogenase activity in mitochondria with Ca^{2+} and Sr^{2+} were $0.54 \pm 0.02\,\mu M$ and $7.05 \pm 1.25\,\mu M$ respectively (16 observations in both cases).

Effects of Ca^{2+} and Sr^{2+} on the activity of NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase in extracts of mitochondria from rat interscapular brown adipose tissue

Table 1 summarizes the kinetic properties of NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase activity observed at pH 7.2 in extracts of brown-adipose-tissue mitochondria. The properties were very similar to those published previously for the rat heart NAD^+–isocitrate dehydrogenase (Denton *et al.*, 1978) and pig heart oxoglutarate dehydrogenase (McCormack & Denton, 1979). Features pertinent to the present paper were as follows.

(*a*) NAD^+–isocitrate dehydrogenase exhibited sigmoidal kinetics with respect to its substrate; in the presence of $20\,\mu M$-Ca^{2+}, the K_m for *threo*-D_s-isocitrate was decreased from 0.58 to 0.14 mM, but there was little change in the $V_{max.}$. At a low concentration of *threo*-D_s-isocitrate (0.1 mM) the calculated $k_{0.5}$ for activation by Ca^{2+} was about

Table 1. *Kinetic parameters of NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase in extracts of rat brown-adipose-tissue mitochondria*

Mitochondrial extracts were prepared as described in the Experimental section. Enzyme activities were assayed at pH 7.2 and 30°C in the presence of 2 mM-NAD^+ and 1 mM-$MgCl_2$ plus 1 mM-thiamin pyrophosphate and 0.25 mM-CoA for oxoglutarate dehydrogenase. When added, ADP was 1 mM. The concentrations of Ca^{2+} and Sr^{2+} were varied by using 5 mM-EGTA buffers. For the determination of $k_{0.5}$ for Ca^{2+} and Sr^{2+} the concentration of substrate was 0.1 mM (first two columns) or 0.05 mM (third column). Values are derived from observations on two to four different preparations of mitochondria which were fitted to either $v = V_{max.}/\{1 + (K_m/[S])^n\}$ or $v = (V_{max.}/\{1 + (k_{0.5}/[M^{2+}])^n\}) + V_0$, where V_0 is the rate in the absence of added Ca^{2+} or Sr^{2+}. Values are expressed as means ± S.E.M. for the numbers of degrees of freedom shown in parentheses.

		Parameter values for		
		NAD^+–isocitrate dehydrogenase	Oxoglutarate dehydrogenase	
Condition	Parameter	(+ADP)	(−ADP)	(+ADP)
5 mM-EGTA ($[Ca^{2+}] < 1$ nM)	K_m for substrate (mM)	0.48 ± 0.020 (14)	1.48 ± 0.26 (12)	0.15 ± 0.01 (14)
	n	3.1 ± 0.31 (14)	1.1 ± 0.19 (12)	1.1 ± 0.12 (14)
	$V_{max.}$ (units/mg of protein)	0.13 ± 0.004 (14)	0.15 ± 0.01 (12)	0.13 ± 0.006 (14)
5 mM-EGTA plus 5 mM-$CaCl_2$ ($[Ca^{2+}] \simeq 20\,\mu M$)	K_m for substrate (mM)	0.14 ± 0.005 (14)	0.12 ± 0.017 (15)	0.026 ± 0.004 13)
	n	2.7 ± 0.019 (14)	1.0 ± 0.12 (15)	1.3 ± 0.19 (13)
	$V_{max.}$ (units/mg of protein)	0.14 ± 0.003 (14)	0.16 ± 0.006 (15)	0.15 ± 0.005 (13)
0.05 or 0.1 mM substrate	$k_{0.5}$ for Ca^{2+} (μM)	1.6 ± 0.19 (17)	0.76 ± 0.13 (15)	0.21 ± 0.06 (14)
	$k_{0.5}$ for Sr^{2+} (μM)	15.8 ± 2.04 (16)	9.6 ± 0.58 (15)	3.33 ± 0.46 (10)

1.6μM; Sr^{2+} has similar effects to Ca^{2+}, but the $k_{0.5}$ was about 10 times greater.

(*b*) Oxoglutarate dehydrogenase exhibited hyperbolic kinetics with respect to its substrate; both Ca^{2+} and ADP lowered the K_m for oxoglutarate. Ca^{2+} (20μM) markedly lowered the K_m both in the absence of ADP (from 1.5 to 0.12mM) and in the presence of 1mM-ADP (from 0.15 to 0.03mM). At low concentrations of oxoglutarate (0.1 or 0.05mM), the $k_{0.5}$ for the activation by Ca^{2+} was 0.8μM in the absence of ADP and 0.2μM in the presence of ADP. Sr^{2+} had similar effects to Ca^{2+}, but the values for the $k_{0.5}$ were about 10 times greater both in the absence and in the presence of ADP. The increased sensitivity to Ca^{2+} in the presence of ADP has also been found for the rat heart enzyme (Denton *et al.*, 1980).

The K_m of both NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase in extracts of brown-adipose-tissue mitochondria for their respective substrates was also found to be decreased with decreasing pH (results not shown). This had been observed previously for the two enzymes from pig heart (Colman, 1975; McCormack & Denton, 1979).

Effects of Ca^{2+} and Sr^{2+} on the oxidation of threo-D_s-isocitrate, oxoglutarate and other substrates by uncoupled rat brown-adipose-tissue mitochondria

Brown-adipose-tissue mitochondria were incubated in KCl-based medium containing Mg^{2+}, phosphate and malate. Under these conditions, there was little or no O_2 uptake until an oxidizable substrate such as *threo*-D_s-isocitrate or oxoglutarate or succinate (with no malate) was added. Fig. 2 shows the time courses of O_2 uptake observed in a typical experiment in which the effects of the presence of Ca^{2+} (about 20μM) in the incubation medium on the rates of oxidation of *threo*-D_s-isocitrate, oxoglutarate and succinate were explored. At a low concentration (0.1mM), the rate of oxidation of both *threo*-D_s-isocitrate and oxoglutarate was 4–5 times greater in mitochondria incubated with Ca^{2+} than in those incubated with EGTA; there was no such difference at a high concentration of *threo*-D_s-isocitrate (2mM) and oxoglutarate (5mM). In contrast, the presence of 20μM-Ca^{2+} had no effect on succinate oxidation at either a low or saturating concentration and also no effect on the oxidation of ascorbate in the presence of *NNN'N'*-tetramethyl-*p*-phenylenediamine. These observations suggested that Ca^{2+} may decrease the K_m values for the oxidation of *threo*-D_s-isocitrate and oxoglutarate to much the same extent as that found with NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase in mitochondrial extracts.

This raised the possibility that the properties of

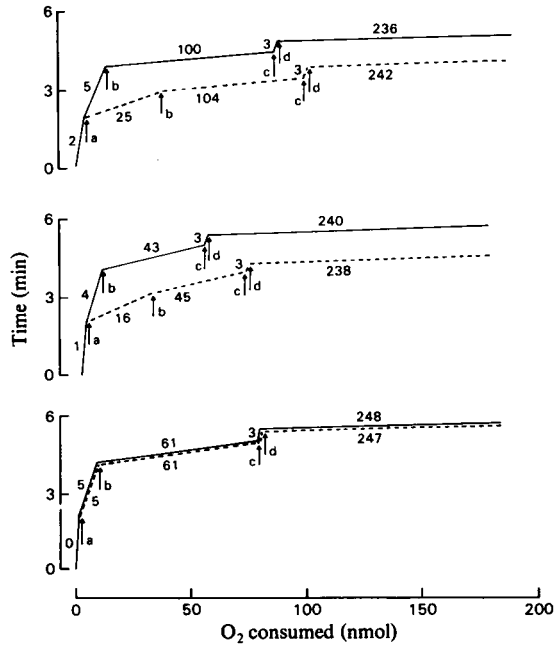

Fig. 2. *Effects of Ca^{2+} on the uptake of oxygen by uncoupled brown-adipose-tissue mitochondria*

Mitochondria (0.5mg of protein) were incubated at 30°C in 1ml of 125mM-KCl/20mM-Tris/HCl pH 7.2, containing 5mM-KH_2PO_4, 0.5mM-$MgCl_2$ and (except in lower panel) 1mM-L-malate together with either 5mM-EGTA (solid lines; $[Ca^{2+}] < 1$nm) or 5mM-EGTA plus 5mM-$CaCl_2$ (broken lines; $[Ca^{2+}] \simeq 20\mu$M). Further additions at the times indicated were made as follows. Upper panel: a, 0.1mM-*threo*-D_s-isocitrate; b, 3mM-*threo*-D_s-isocitrate; c, antimycin (0.5μg/ml); d, 20mM-ascorbate plus 0.5mM-*NNN'N'*-tetramethylphenylenediamine. Centre panel; a, 0.1mM-2-oxoglutarate; b, 5mM-2-oxoglutarate; c and d, as for top traces. Lower panel: a, 0.1mM-succinate; b, 10mM-succinate; c and d, as for top traces. The values given on the traces indicate nmol of O_2 consumed/min per mg of protein.

NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase could be studied in these mitochondria by simply following rates of O_2 uptake at various extramitochondrial concentrations of substrate and Ca^{2+}. The maximum rates of O_2 uptake in the presence of *threo*-D_s-isocitrate and oxoglutarate were about 0.15 and 0.07μmol/min per mg of protein respectively. It is unlikely that the oxidation of either *threo*-D_s-isocitrate or oxoglutarate is limited by the capacity of the respiratory chain to oxidize NADH, as much higher rates of O_2 uptake can be achieved in the presence of pyruvate (maximum rate about 0.30μmol/min per mg of protein) and palmitoylcarnitine (maximum rate about 0.21μmol/min per mg of protein). The mitochondria appeared to be

in a fully uncoupled state, as the addition of the uncoupler FCCP (0.5 μM) had no perceptible effect on the rates of O_2 uptake at either low or saturating concentrations of *threo*-D_s-isocitrate, oxoglutarate or succinate. In the experiment shown in Fig. 2, the mitochondria were preincubated in medium containing EGTA or CaEGTA buffer for 2 min before addition of substrate to allow equilibration of extramitochondrial and intramitochondrial Ca^{2+}. Equilibration in fact appeared to be achieved within 30 s, as no differences were observed between mitochondria preincubated for periods of 30 s up to 5 min. Moreover, the addition of the ionophore A23187 (2 μg/ml) had no effect on the sensitivity of the oxidation of either *threo*-D_s-isocitrate or oxoglutarate to extramitochondrial Ca^{2+} (results not shown).

Effects of varying the concentration of *threo*-D_s-isocitrate, citrate, oxoglutarate and succinate on rates of O_2 uptake in presence and absence of Ca^{2+} (about 20 μM) are shown in Fig. 3. Kinetic constants calculated from these observations are given in Table 2(*a*). The results confirmed that Ca^{2+} over this range of concentrations had no effect on succinate oxidation, but greatly decreased the K_m values for the oxidation of *threo*-D_s-isocitrate and oxoglutarate. The presence of Ca^{2+} also decreased the K_m for the oxidation of citrate, but in addition there was also a small increase in the $V_{max.}$. The effect of Ca^{2+} on the oxidation of palmitoyl-L-carnitine was also investigated, but, as with the oxidation of succinate, no changes were observed (results not shown).

The effects of changes in the concentration of Ca^{2+} and Sr^{2+} on the oxidation of *threo*-D_s-isocitrate and oxoglutarate when these substrates were present in the mitochondrial incubation medium at 0.1 mM are shown in Fig. 4 and the calculated $k_{0.5}$ values are given in Table 3(*a*). Over the range of Ca^{2+} and Sr^{2+} concentrations studied, there were no changes in the rates of oxidation of *threo*-D_s-

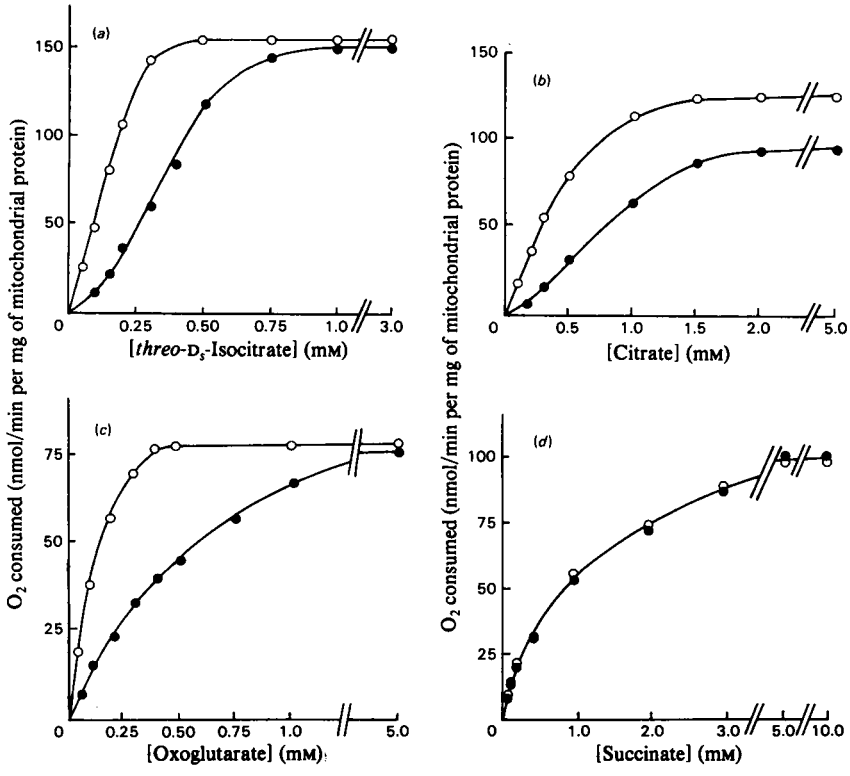

Fig. 3. *Effects of varying the concentrations of* (*a*) *threo*-D_s-*isocitrate*, (*b*) *citrate*, (*c*) *2-oxoglutarate and* (*d*) *succinate on the oxygen uptake by uncoupled brown-adipose-tissue mitochondria incubated in the presence* (○) *or absence* (●) *of* Ca^{2+}

Mitochondria (1 mg of protein) were incubated at 30°C in 1 ml of 125 mM-KCl, 20 mM-Tris/HCl, pH 7.2, containing 5 mM-KH_2PO_4, 0.5 mM-$MgCl_2$, 1 mM-L-malate [not added in (*d*)], together with either 5 mM-EGTA (●, $[Ca^{2+}] < 1$ nM) or 5 mM-EGTA plus 5 mM-$CaCl_2$ (○, $[Ca^{2+}] \simeq 20$ μM) and the indicated concentrations of substrates. Each point represents the mean of observations made on at least three different preparations of mitochondria; calculated kinetic parameters are given in Table 2(*a*).

Table 2. *Summary of kinetic parameters for the oxidation of threo-D_s-isocitrate, oxoglutarate, citrate and succinate by uncoupled brown-adipose-tissue mitochondria*

(*a*) Individual observations of the results shown in Fig. 3 were fitted to the equation $v = V_{max.}/\{1 + (K_m/[S])^n\}$. Derived values for $V_{max.}$, n and K_m are given as means ± S.E.M. for the numbers of degrees of freedom shown in parentheses. Values for $V_{max.}$ are expressed as μmol of O_2 taken up/min per mg of protein. (*b*) Values derived from parallel experiments to those in (*a*), except that albumin (1 mg/ml) was present in the incubation medium. *$P < 0.01$ for effect of Ca^{2+} versus EGTA control (Student's *t* test).

Condition	Parameter	Parameter values for the oxidation of: *threo*-D_s-Isocitrate	Oxoglutarate	Citrate	Succinate
(*a*)					
5 mM-EGTA	K_m (mM)	0.41 ± 0.012 (45)	0.48 ± 0.038 (37)	0.89 ± 0.049 (18)	0.99 ± 0.07 (28)
($[Ca^{2+}] < 1$ nM)	n	2.2 ± 0.15 (45)	1.2 ± 0.10 (37)	2.1 ± 0.22 (18)	0.92 ± 0.06 (28)
(control)	$V_{max.}$	0.15 ± 0.004 (45)	0.069 ± 0.002 (37)	0.096 ± 0.004 (18)	0.092 ± 0.003 (28)
5 mM-EGTA plus	K_m (mM)	0.15 ± 0.009* (42)	0.11 ± 0.010* (28)	0.37 ± 0.02* (18)	0.97 ± 0.076 (27)
5 mM-$CaCl_2$	n	2.1 ± 0.21 (42)	1.5 ± 0.20 (28)	1.8 ± 0.14 (18)	0.96 ± 0.08 (27)
($[Ca^{2+}] \simeq 20$ μM)	$V_{max.}$	0.16 ± 0.007 (42)	0.076 ± 0.003 (18)	0.14 ± 0.004 (18)	0.096 ± 0.003 (27)
(*b*)					
Albumin (1 mg/ml),	K_m (mM)	0.14 ± 0.009 (33)	0.15 ± 0.012 (15)	0.36 ± 0.04 (7)	0.34 ± 0.046 (16)
5 mM-EGTA	n	1.9 ± 0.22 (33)	1.5 ± 0.18 (15)	1.9 ± 0.3 (7)	1.3 ± 0.16 (16)
($[Ca^{2+}] < 1$ nM)	$V_{max.}$	0.15 ± 0.006 (33)	0.074 ± 0.002 (15)	0.10 ± 0.007	0.11 ± 0.005 (16)
(control)					
Albumin (1 mg/ml),	K_m (mM)	0.056 ± 0.004* (22)	0.061 ± 0.08* (13)	0.13 ± 0.011* (7)	0.33 ± 0.049 (15)
5 mM-EGTA plus	n	1.83 ± 0.21 (22)	1.64 ± 0.38 (13)	2.7 ± 0.62 (7)	1.41 ± 0.17 (15)
5 mM-$CaCl_2$	$V_{max.}$	0.15 ± 0.005 (22)	0.078 ± 0.004 (13)	0.14 ± 0.005 (7)	0.11 ± 0.009 (15)
($[Ca^{2+}] \simeq 20$ μM)					

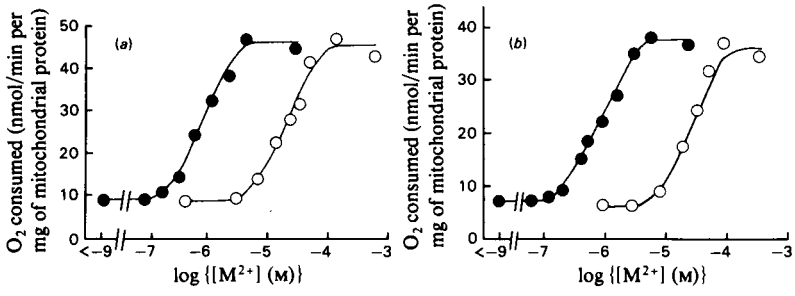

Fig. 4. *Sensitivity to Ca^{2+} and Sr^{2+} of the oxidation of (a) threo-D_s-isocitrate (0.1 mM) and (b) 2-oxoglutarate (0.1 mM) by uncoupled brown-adipose-tissue mitochondria*

Mitochondria were incubated as described in Fig. 3 in the presence of 5 mM-EGTA and additions of $CaCl_2$ (●) or $SrCl_2$ (○) to give the appropriate concentration of free metal ions. Each point represents the mean of observations on two to six different preparations of mitochondria; calculated kinetic parameters are given in Table 3(*a*).

isocitrate and oxoglutarate when saturating concentrations of the substrates were present.

A comparison of the kinetic constants for NAD^+–isocitrate dehydrogenase studied in the presence of ADP in samples of mitochondrial extracts (Table 1) and the oxidation of *threo*-D_s-isocitrate by uncoupled brown-adipose-tissue mitochondria (Tables 2*a* and 3*a*) shows striking similarities. In both cases the kinetics with varying concentrations of *threo*-D_s-isocitrate were sigmoidal, with Ca^{2+} decreasing the K_m for *threo*-D_s-isocitrate from close to 0.5 mM to about 0.15 mM with no effect on the $V_{max.}$. The $k_{0.5}$ for Ca^{2+} at 0.1 mM-*threo*-D_s-isocitrate was about 1 μM, and the effects of Ca^{2+} were mimicked by Sr^{2+}, but the $k_{0.5}$ was at least an order of magnitude greater. There are also close qualitative similarities between the oxidation of oxoglutarate by the intact mitochondria and the properties of oxoglutarate dehydrogenase in mitochondrial extracts. The kinetics with varying concentrations of oxoglutarate were essentially hyperbolic, and Ca^{2+} decreased the K_m for oxoglutarate by more than 75%. Both the K_m

Table 3. *Summary of the kinetic parameters for the activation by Ca^{2+} and Sr^{2+} of the oxidation of threo-D_s-isocitrate (0.1 mM) and oxoglutarate (0.1 mM) by uncoupled brown-adipose-tissue mitochondria*

(*a*) Individual observations of the results shown in Fig. 4 were fitted to the equation $v = (V_{max.}/\{1+(k_{0.5}/[M^{2+}])^n\}) + V_0$, where V_0 is the activity in the absence of Ca^{2+} or Sr^{2+}. Derived values for the $k_{0.5}$ for Ca^{2+} and Sr^{2+} are given as means ± S.E.M. for the numbers of degrees of freedom shown in parentheses. (*b*) Values derived from parallel experiments to those in (*a*), except that albumin (1 mg/ml) was present in the incubation medium.

		Parameter values for the activation of	
Condition	Parameter	*threo*-D_s-Isocitrate oxidation	Oxoglutarate oxidation
(*a*) 0.1 mM substrate	$k_{0.5}$ for Ca^{2+} (μM)	0.96 ± 0.17 (28)	0.86 ± 0.18 (16)
	$k_{0.5}$ for Sr^{2+} (μM)	17.2 ± 2.2 (38)	19.4 ± 1.6 (17)
(*b*) 50 μM substrate + albumin (1 mg/ml)	$k_{0.5}$ for Ca^{2+} (μM)	0.30 ± 0.077 (24)	0.39 ± 0.08 (16)
	$k_{0.5}$ for Sr^{2+} (μM)	5.6 ± 0.96 (19)	7.6 ± 1.6 (8)

value for oxoglutarate in the presence of 20 μM-Ca^{2+} and the $k_{0.5}$ value for the effect of Ca^{2+} at 0.1 mM-oxoglutarate were close to the corresponding values found for the extracted enzyme assayed in the absence of ADP. However, the K_m for oxoglutarate oxidation in the absence of Ca^{2+} was not as high as that found with the isolated enzyme.

The rates of O_2 uptake at saturating concentrations of *threo*-D_s-isocitrate and oxoglutarate were approx. 0.15 and 0.075 μmol of O_2/min per mg of protein (Table 2). This is equivalent to the transfer to the respiratory chain of reducing power from 0.30 and 0.15 μmol of NADH formed/min per mg of protein respectively. These results are compatible with succinate being the major end product for the oxidation of both *threo*-D_s-isocitrate and oxoglutarate by the brown-adipose-tissue mitochondria, so that 2 mol of NADH are formed per mol of *threo*-D_s-isocitrate oxidized, but only 1 in the oxidation of oxoglutarate.

Further evidence for this view came from the measurement of intramitochondrial concentrations of ATP associated with the oxidation of *threo*-D_s-isocitrate, oxoglutarate and succinate (results not shown). When mitochondria were oxidizing succinate in the presence or absence of Ca^{2+}, there was little or no intramitochondrial ATP (<0.1 nmol/mg of protein), as expected in uncoupled mitochondria. However, ATP was formed in the presence of either *threo*-D_s-isocitrate or oxoglutarate, presumably via the substrate-level phosphorylation of GDP by succinyl-CoA synthetase (EC 6.2.1.4). With increasing concentrations of *threo*-D_s-isocitrate or oxoglutarate, the ATP content of the mitochondria increased in parallel with the increases in rates of oxidation. Values for ATP content after 1 min of incubation with any particular concentration of substrate were very similar to values obtained after 5 min, and thus the ATP content appeared quickly to reach a steady-state value. At saturating concentrations of *threo*-D_s-isocitrate or oxoglutarate, the presence of Ca^{2+} had no effect on the mitochondrial ATP content (about 1.5 nmol/mg of protein). However, at non-saturating concentrations of the substrates the ATP contents were significantly increased in the presence of Ca^{2+} (20 μM) in line with the increased rates of oxidation of *threo*-D_s-isocitrate and oxoglutarate caused by Ca^{2+}. For example, at 0.1 mM-*threo*-D_s-isocitrate the ATP contents after 1 min incubation in the absence and presence of Ca^{2+} were 0.31 ± 0.07 and 0.88 ± 0.14 nmol/mg of protein respectively (means ± S.E.M. for three observations in each case). The maximum values for ATP content in the uncoupled mitochondria were less than 50% of those reached in coupled mitochondria incubated with albumin and GDP (3–4 nmol/mg of protein).

All the studies described above have been carried out at pH 7.2. The K_m values for both NAD^+–isocitrate dehydrogenase (Colman, 1975) and oxoglutarate dehydrogenase (McCormack & Denton, 1979) for their respective substrates are rather sensitive to changes in pH. The effects of changing the pH from 7.2 to 7.0 on the activity of these enzymes in mitochondrial extracts were compared with the effects of this change in pH on the oxidation of *threo*-D_s-isocitrate and oxoglutarate by the intact uncoupled mitochondria (results not shown). In both instances the changes in pH had no effect at saturating concentrations of the two substrates, but resulted in increases in rates at low concentrations of the substrates. The oxidation of succinate was essentially unaffected by the change in pH (results not shown).

Effects of albumin on the oxidation of threo-D_s-isocitrate, oxoglutarate, citrate and succinate by rat brown-adipose-tissue mitochondria

Addition of albumin to the incubation medium of brown-adipose-tissue mitochondria from hamsters or guinea pigs has been shown to result in a modest increase in the protonmotive force (see Nicholls,

1979). Results given in Table 2(*b*) indicate that the apparent K_m values for the oxidation of *threo*-D_s-isocitrate, oxoglutarate, citrate and also succinate are all decreased by about 50–80% in both the absence and the presence of 20μM-Ca^{2+}. This is compatible with the presence of a small pH gradient across the mitochondrial inner membrane under these conditions, resulting in some accumulation of the substrates and thus the decrease in apparent K_m values.

The sensitivity of oxidation of *threo*-D_s-isocitrate and oxoglutarate at low concentration of the substrates (0.05mM) to Ca^{2+} and Sr^{2+} is also increased by a factor of about 3 when albumin was added to the incubation medium (Table 3*b*). Under the conditions of these studies, no respiratory control was apparent in the presence of albumin.

General discussion and conclusions

The data presented here show that there is a close similarity between the oxidation of *threo*-D_s-isocitrate and oxoglutarate by uncoupled brown-adipose-tissue mitochondria and the properties of NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase respectively in mitochondrial extracts. This suggests that the enzyme activities are limiting in the oxidation of these substrates.

Rat brown-adipose-tissue mitochondria contain $NADP^+$–isocitrate dehydrogenase with a maximum activity comparable with that of NAD^+–isocitrate dehydrogenase. The NADP-linked enzyme is similar to that from heart mitochondria, has a K_m for *threo*-D_s-isocitrate less than 5μM and is insensitive to Ca^{2+} (Denton *et al.*, 1978; R. M. Denton, unpublished work). Since the kinetics and properties of *threo*-D_s-isocitrate oxidation resemble so closely those of the NAD-linked enzyme, it follows that the NADP-linked enzyme plays little or no role in the oxidation of *threo*-D_s-isocitrate under these conditions, presumably because the transfer of reducing equivalents from NADPH to the respiratory chain is very slow (Nicholls & Garland, 1969; Plaut & Smith, 1977).

The K_m values of NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase for their substrates decrease with increases in [ADP] and $[H^+]$. However, it is most unlikely that the decrease in apparent K_m for oxidation of *threo*-D_s-isocitrate and oxoglutarate observed in the mitochondria incubated with Ca^{2+} was the result of a decrease in the intramitochondrial ATP/ADP concentration ratio and/or pH. Under conditions where Ca^{2+} stimulated the oxidation of *threo*-D_s-isocitrate or oxoglutarate, the ATP content was actually increased (results given in the text). Moreover, the mitochondria behaved as if fully uncoupled, and the addition of FCCP did not alter oxidation rates or Ca^{2+}-sensitivity. If Ca^{2+} were to elicit changes in the gradient of H^+ ions across the mitochondrial inner membrane, then the distribution of succinate across the membrane should also be affected and thus result in a diminution in the apparent K_m for succinate oxidation: no such change was seen in the uncoupled mitochondria (Table 2*a*). However, when albumin was present, which allows a small pH gradient to be maintained, then a modest change in the K_m for succinate was observed (Table 2*b*).

Our earlier studies (see the introduction) have shown that pyruvate dehydrogenase phosphate phosphatase, NAD^+–isocitrate dehydrogenase and oxoglutarate dehydrogenase activities in mitochondrial extracts are all activated by Ca^{2+}, with $k_{0.5}$ values of the order of 1μM, and by Sr^{2+}, with $k_{0.5}$ values some 10 times higher. The present studies now allow the conclusion that all these enzymes maintain their sensitivity to Ca^{2+} and Sr^{2+} when located within white or brown-adipose tissue mitochondria. The following paper (Denton *et al.*, 1980) offers evidence that the pyruvate dehydrogenase phosphate phosphatase and oxoglutarate dehydrogenase in intact heart mitochondria also maintain this sensitivity to Ca^{2+}, and it would seem reasonable to expect that this will hold true for other mammalian mitochondria.

We suggested previously (McCormack & Denton, 1979) that it may be possible to use the Ca^{2+}-sensitive enzymes as a means of assessing intramitochondrial Ca^{2+} concentrations. In the present studies a small membrane potential was set up across the mitochondrial inner membrane by adding albumin, and under these circumstances some accumulation of Ca^{2+} within the mitochondria would be expected. In agreement with this, the apparent $k_{0.5}$ for both Ca^{2+} and Sr^{2+} on oxidation of *threo*-D_s-isocitrate and oxoglutarate was diminished by about 3-fold, indicating that the Ca^{2+} concentration within the mitochondria incubated with albumin was approx. 3 times the concentration in the incubation medium.

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