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Calcium Ions and the Regulation of NAD$^+$-Linked Isocitrate Dehydrogenase from the Mitochondria of Rat Heart and Other Tissues

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The effects of Ca^{2+} on the activity of isocitrate dehydrogenase (NAD$^+$) in extracts of rat heart mitochondria were explored in the presence of $MgCl_2$ by using EGTA buffers. In the absence of ADP, Ca^{2+} (about 30 μM) resulted in a slight increase in apparent K_m for *threo*-D_s-isocitrate; in the presence of ADP, Ca^{2+} (about 25 μM) greatly lowered the apparent K_m for *threo*-D_s-isocitrate from 227 μM to 53 μM without changing the maximum velocity. At 100 μM-*threo*-D_s-isocitrate and 1 mM-ADP, there was an 8-fold activation by Ca^{2+}, with a K_m for Ca^{2+} of 1.2 μM. This activation was also observed with Sr^{2+} (K_m 3.1 μM), but not with Mn^{2+} (at concentrations below 2.5 μM). Similar effects of Ca^{2+} were also observed on isocitrate dehydrogenase (NAD$^+$) activity in extracts of mitochondria from liver, kidney, brown adipose tissue and white adipose tissue of the rat. The possible regulatory role of changes in the intramitochondrial concentration of Ca^{2+} is discussed.

Pyruvate dehydrogenase phosphate phosphatase extracted from the mitochondria of a number of mammalian tissues, including pig and rat heart, is activated by Ca^{2+} ions with a K_m of about 2 μM (Denton *et al.*, 1972, 1975). Evidence has also been obtained, by using fat-cell mitochondria incubated in the presence of the bivalent-metal-ionophore A23187, that the phosphatase within intact mitochondria is sensitive to changes in the intramitochondrial concentration of Ca^{2+} (Severson *et al.*, 1974). In addition, pyruvate dehydrogenase kinase, at least from pig heart, is partially inhibited by Ca^{2+} at concentrations above 0.1 μM (Cooper *et al.*, 1974). It is thus well established that the proportion of the pyruvate dehydrogenase complex in the active, non-phosphorylated form may be increased in heart and other mammalian tissues on increasing the intramitochondrial Ca^{2+} concentration (but only if the change occurs below 10 μM).

Therefore it seemed paradoxical that NAD-ICDH from rat heart mitochondria should be inhibited by Ca^{2+} (Vaughan & Newsholme, 1969; Zammit & Newsholme, 1976). Other studies (Chen & Plaut, 1963; Goebell & Klingenberg, 1964; Stein *et al.*, 1967; Plaut, 1970; Colman, 1975) have shown that NAD-ICDH from mammalian sources requires Mn^{2+} or Mg^{2+}, that the activity of the enzyme increases sigmoidally with increasing concentrations of its substrate, *threo*-D_s-isocitrate, and that the apparent K_m for *threo*-D_s-isocitrate is greatly diminished in the presence of ADP. Newsholme and his colleagues (Vaughan & Newsholme, 1969; Zammit & Newsholme, 1976) concluded from their studies that Ca^{2+} increased the apparent K_m of the rat heart enzyme for *threo*-D_s-isocitrate in both the presence and the absence of ADP. They conducted their studies in the presence of 5 mM-$MnCl_2$ and regulated the free Ca^{2+} concentration by adding 1 mM-EGTA and calculated amounts of $CaCl_2$. However, the binding of Mn^{2+} to EGTA seems to have been neglected, although the affinity of EGTA for Mn^{2+} is greater than that for Ca^{2+} (Sillén *et al.*, 1971). It can be calculated that with 1 mM-EGTA and 5 mM-$MnCl_2$ at pH 7.0, over 99% of EGTA will be as MnEGTA. We suspect that the range of free Ca^{2+} concentrations studied by Vaughan & Newsholme (1969) and Zammit & Newsholme (1976) was between 10 μM and 1 mM rather than between 1 nM and 10 μM as the authors assumed.

We have re-examined the sensitivity of NAD-ICDH from the mitochondria of rat heart and other tissues to changes in Ca^{2+} concentrations, but $MnCl_2$ has been replaced by $MgCl_2$, since Mg^{2+} is bound very weakly by EGTA compared with Mn^{2+} or Ca^{2+}. The present paper reports that micromolar concentrations of Ca^{2+} greatly decrease the apparent K_m for *threo*-D_s-isocitrate at saturating concentrations of Mg^{2+}, ADP and NAD$^+$. This effect is mimicked by Sr^{2+}, but not Mn^{2+}. In the absence of ADP, there may be a small inhibitory effect of Ca^{2+} ions.

Abbreviations used: NAD-ICDH, NAD$^+$-linked isocitrate dehydrogenase (EC 1.1.1.41); NADP-ICDH, NADP$^+$-linked isocitrate dehydrogenase (EC 1.1.1.42).

Experimental

Chemicals and biochemicals

Except where stated otherwise, all enzymes, coenzymes and substrates were from Boehringer Corp. (London) Ltd., Lewes, East Sussex BN7 2DF, U.K.,

and all other chemicals, including EGTA, were the highest grade available from BDH Chemicals, Poole, Dorset, U.K.

DL-Isocitrate (trisodium salt) was from Sigma (London) Chemical Co. Ltd., Poole, Dorset BH17 7NH, U.K., and was shown to contain 50% (w/w) *threo*-D_s-isocitrate by enzymic assay with isocitrate dehydrogenase ($NADP^+$) (Siebert, 1965). *threo*-D_s-Isocitrate (monopotassium salt) was prepared by the method of Vickery (1963). After recrystallization from hot water the final product contained over 95% (w/w) monopotassium *threo*-D_s-isocitrate by enzymic assay; contamination by L-malate was checked by enzymic assay (Hohorst, 1965) and found to be less than 0.05%.

Preparation of extracts of mitochondria

Mitochondria were prepared from the tissues of fed male or female rats (200–300g) by the following methods: heart (Kerbey *et al.*, 1976), liver (Chappell & Hansford, 1972) and epididymal white adipose tissue (Severson *et al.*, 1976). Kidney mitochondria were prepared as for heart. Mitochondria from brown adipose tissue were prepared from interscapular tissue of rats cold-adapted for 4 weeks at 5°C by a slight modification of the method for epididymal white adipose tissue. In all cases, samples of the intact mitochondria (2–4mg of protein) were sedimented by centrifugation in an Eppendorf 3200 Minicentrifuge at about 10000*g* for 1min and extracted by freezing and thawing three times in 500μl of extraction buffer (100mM-potassium phosphate, pH7.2, containing 1mM-ADP and 5mM-2-mercaptoethanol). The extract was centrifuged for 5min at 10000*g* in the Eppendorf 3200 instrument and the supernatant used for the study of NAD-ICDH activity. The pellet contained less than 5% of the activity in the supernatant.

Assay of NAD-ICDH activity

Activity was measured by following the change in A_{340} at 30°C by using a Gilford recording spectrophotometer (model 240). The assay medium was 50mM-Mops (4-morpholinepropanesulphonic acid), pH7.0, plus 2μg of rotenone/ml containing additions of $MgCl_2$, $MnCl_2$, $SrCl_2$, $CaCl_2$, EGTA, isocitrate, NAD^+ and ADP as indicated. Assays were conducted in a total volume of 1.5ml and were initiated by addition of mitochondrial extract (5–20μl). Rates were essentially linear with time for at least 3min and the initial rates were also linear with volume of extract over the range used. Rates in the absence of added isocitrate or NAD^+ were less than 1% of maximal rates. The addition of rotenone was essential to inhibit the NADH oxidase activity that remained in the supernatant after centrifugation of mitochondrial extracts. In the absence of rotenone, the activity of NADH oxidase measured in centrifuged extracts of rat heart mitochondria under the conditions of the assay for NAD-ICDH plus NADH (40μM) was 150–200 munits/mg of mitochondrial protein; in the presence of rotenone (2μg/ml) this was decreased to less than 2.5 munits/mg of protein. Aconitate hydratase activity was present in the extracts at about the same activity as NAD-ICDH, but did not appear to interfere to any appreciable extent with the measurement of initial rates of NAD-ICDH activity. Essentially similar kinetic behaviour was found with a partially purified preparation of rat heart NAD-ICDH freed of aconitate hydratase activity by poly(ethylene glycol) fractionation of a mitochondrial extract.

Use of EGTA buffers and calculation of the concentration of bivalent metal ions

Free concentrations of Ca^{2+}, Sr^{2+} and, in some cases, Mn^{2+} were controlled by the use of EGTA buffers (Portzehl *et al.*, 1964). Stock solutions were prepared of 150mM-EGTA and of 150mM-EGTA containing 150mM-$CaCl_2$ or 150mM-$SrCl_2$ or 150mM-$MnCl_2$, all adjusted to pH7.0 with KOH. Calculated proportions were then added to give 5mM-EGTA and the desired free concentration of the appropriate metal ion. This procedure was adopted rather than one using separate stock solutions of EGTA and the metal chloride, since the release of protons that accompanies the binding of bivalent metal ions to EGTA is minimized. Particular care was taken that the stock solutions of EGTA containing $CaCl_2$ were equimolar in total calcium and EGTA. Three methods were used and found to give essentially similar results. The methods were: (*a*) to follow the release of protons on adding $CaCl_2$ to EGTA at pH7.0 by using a pH-meter; (*b*) to measure free Ca^{2+} by using Arsenazo III [2-(*o*-arsonophenylazo)-1,8-dihydroxynaphthalene-3,6-disulphonic acid]; and (*c*) to measure free Ca^{2+} by using a Ca^{2+}-selective poly(vinyl chloride) electrode (Ammann *et al.*, 1975), kindly made available by Professor J. B. Chappell of this Department. Method (*a*) was also used to check that the stock solutions of EGTA containing $MnCl_2$ were equimolar.

Concentrations of free bivalent-metal ions in the presence of EGTA, ADP and isocitrate were computed by the program of Feldman *et al.* (1972) adapted for use with a Hewlett–Packard 9821 computer by Dr. Paul England of this Department. Apparent dissociation constants at pH7.0 used in these calculations are given in Table 1. The weak binding of the bivalent metal ions to NAD^+ was neglected (Colman, 1972).

Table 1. *Apparent dissociation constants at pH 7.0*

The constants (M) were calculated from stability constants and pK values measured at 25 or 30°C (Martell & Sillén, 1964; Sillén *et al.*, 1971; Grzybowski *et al.*, 1970) as described by Portzehl *et al.* (1964).

Ligand	Bivalent metal ion ...	Apparent dissociation constant (M)			
		Mg^{2+}	Ca^{2+}	Sr^{2+}	Mn^{2+}
Isocitrate		2.01×10^{-3}	3.56×10^{-3}	1.00×10^{-2}	9.17×10^{-4}
EGTA		2.45×10^{-2}	2.07×10^{-7}	6.40×10^{-5}	1.04×10^{-8}
ADP		1.12×10^{-3}	1.73×10^{-3}	3.57×10^{-3}	8.81×10^{-5}

Enzyme activities and calculation of kinetic constants

Enzyme activities are given as munits/mg of mitochondrial protein, where a munit of activity is taken as that amount which transforms 1 nmol of substrate/min at 30°C. Protein was determined on samples of the pellet of intact mitochondria by a modified biuret method (Gornall *et al.*, 1949), standardized with bovine serum albumin.

Enzyme kinetic constants were determined by fitting data to either $v = V/\{1+(K_m/[S])\}$ or $v = V/\{1+(K_m/[S])^n\}$ by using a non-linear least-squares regression program written for the Hewlett–Packard 9821 computer by Dr. Paul England of this Department [where K_m is the concentration of substrate (S) that results in a velocity (v) equal to half-maximal velocity, i.e. $V/2$]. Unless values of n are quoted, it should be assumed that data were fitted to the first equation, i.e. that the best fit was found when n was not significantly different from 1.

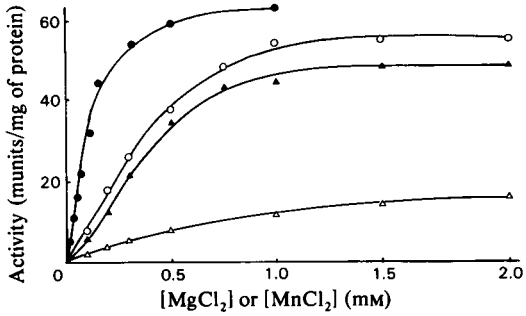

Fig. 1. *Effects of Mg^{2+}, Mn^{2+} and Ca^{2+} on the activity of NAD-ICDH*

Activity was assayed with various concentrations of either $MgCl_2$ (△, ▲, ○) or $MnCl_2$ (●) in the presence of 0.1 mM-*threo*-D_s-isocitrate, 2 mM-NAD^+, 1 mM-ADP and no further additions (○, ●), 5 mM-EGTA (△, Ca^{2+} concentration <1 nM) or 5 mM-EGTA plus 5 mM-$CaCl_2$ (▲, [Ca^{2+}] 26–35 μM).

Results and Discussion

All the results shown in Figures and Tables have been obtained by using purified *threo*-D_s-isocitrate. Parallel studies have also been carried out with the commercial preparation of DL-isocitrate. In all cases virtually identical results were obtained after making allowance for the preparation only containing 50% of *threo*-D_s-isocitrate.

Effects of EGTA on the activity of rat heart NAD-ICDH

Preliminary experiments in the absence of added EGTA or $CaCl_2$ confirmed that NAD-ICDH activity in extracts of rat heart mitochondria was dependent on the presence of either Mn^{2+} or Mg^{2+}, NAD^+ and *threo*-D_s-isocitrate and that the apparent K_m for *threo*-D_s-isocitrate was diminished on addition of ADP. In the presence of 0.1 mM-*threo*-D_s-isocitrate, 2 mM-NAD^+ and 1 mM-ADP, the apparent K_m for $MnCl_2$ was about 0.15 mM compared with 0.40 mM for $MgCl_2$; after allowing for the substantial binding to *threo*-D_s-isocitrate and ADP, the K_m values for the free metal ions were about 10 and 200 μM respectively (Fig. 1). The maximum velocity in the presence of $MgCl_2$ appeared to be about 20% less than that in the presence of $MnCl_2$. The approximate K_m for NAD^+ was 0.1 mM (results not shown); all subsequent studies were carried out at 2 mM to ensure that concentrations of NAD^+ were saturating.

In the presence of 0.1 mM-*threo*-D_s-isocitrate, 1 mM-$MgCl_2$, 1 mM-ADP and 2 mM-NAD^+, addition of EGTA diminished the activity of NAD-ICDH by more than 80%. However, the maximum diminution in activity with EGTA at 1 mM-*threo*-D_s-isocitrate was less than 10% (Fig. 2). The sensitivity to EGTA at 0.1 mM-*threo*-D_s-isocitrate (i.e. half-maximum inhibition at about 50 μM-EGTA) was very similar to that observed previously with pyruvate dehydrogenase phosphate phosphatase (Randle *et al.*, 1974), and is thus compatible with the removal of endogenous Ca^{2+}, which is probably present at a concentration of the order of 10 μM.

The inhibition by 5 mM-EGTA was apparent at all concentrations of Mg^{2+}, but was not evident if the 5 mM-EGTA was replaced by 5 mM-EGTA plus 5 mM-$CaCl_2$ (Fig. 1). Under these latter conditions

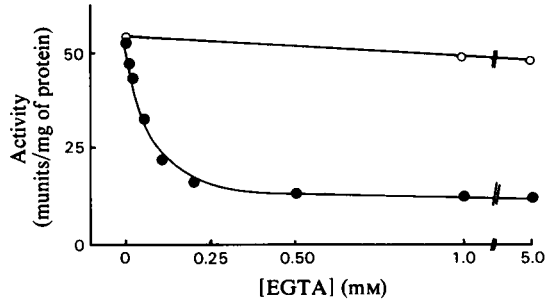

Fig. 2. *Effects of EGTA on the activity of NAD-ICDH*
Activity was assayed with various concentrations of EGTA in the presence of 2mM-NAD^+, 1mM-ADP, 1mM-$MgCl_2$, and either 0.1mM-*threo*-D_s-isocitrate (●) or 1mM-*threo*-D_s-isocitrate (○).

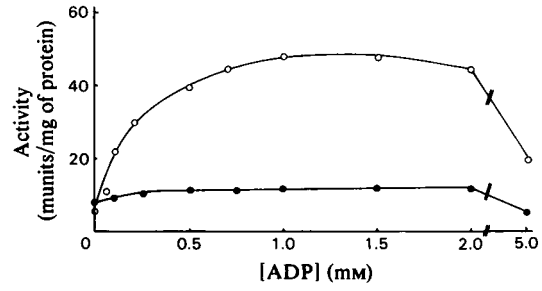

Fig. 3. *Effects of ADP on the activity of NAD-ICDH*
Activity was assayed with various concentrations of ADP in the presence of 0.1mM-*threo*-D_s-isocitrate, 2mM-NAD^+, 1mM-$MgCl_2$ and either 5mM-EGTA (●, $[Ca^{2+}]$ <1nM) or 5mM-EGTA plus 5mM-$CaCl_2$ (○, $[Ca^{2+}]$ 17–32μM).

the free Ca^{2+} concentration varies slightly from 26 to 35μM (depending on the concentration of $MgCl_2$ added).

The effects of 5mM-EGTA are compared with those of 5mM-EGTA plus 5mM-$CaCl_2$ at various concentrations of ADP and *threo*-D_s-isocitrate in Figs. 3 and 4. At low concentrations of *threo*-D_s-isocitrate, there was little or no activation by ADP in the presence of EGTA. On the other hand, in the absence of ADP there was no evidence of any activation by Ca^{2+} ions. In fact the rates with 5mM-EGTA tended to be greater than those with 5mM-EGTA plus 5mM-$CaCl_2$. The data of Fig. 4 were fitted to $v = V/\{1+(K_m/[S])^n\}$ and the values obtained for V, K_m and n are given in Table 2 together with the calculated range of Ca^{2+} concentrations under the four conditions. The values of V and n were essentially unaltered in the presence or absence of Ca^{2+} and/or ADP. The major effect was the substantial

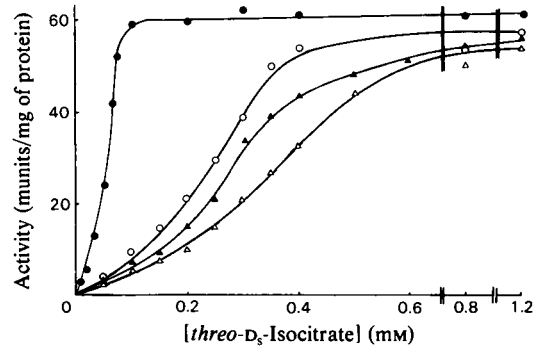

Fig. 4. *Effects of ADP and Ca^{2+} on the affinity of NAD-ICDH for threo-D_s-isocitrate*
Activity was assayed at various concentrations of *threo*-D_s-isocitrate in the presence of 2mM-NAD^+ and 1mM-$MgCl_2$ with the following additions: 5mM-EGTA (▲, Ca^{2+} concentration <1nM), 5mM-EGTA plus 5mM-$CaCl_2$ (△, Ca^{2+} concentration 33–29μM), 5mM-EGTA and 1mM-ADP (○, Ca^{2+} concentration <1nM), 5mM-EGTA plus 5mM-$CaCl_2$ and 1mM-ADP (●, $[Ca^{2+}]$ 28–25μM).

decrease in the value of the K_m for *threo*-D_s-isocitrate in the presence of both ADP and 5mM-EGTA plus 5mM-$CaCl_2$. Under these conditions, the Ca^{2+} concentration varied from 28 to 25μM depending on the isocitrate concentration. There was a small but consistent increase in K_m in the presence of EGTA plus $CaCl_2$ but no added ADP. The effects of free Ca^{2+} concentrations of 1mM were also studied. In the presence of ADP, the effect was very similar to that observed with Ca^{2+} concentrations of about 25μM; in the absence of ADP, the increase in K_m for *threo*-D_s-isocitrate was marginally greater at 1mM than at 25μM-Ca^{2+}. It should be noted that the values of K_m given in Table 2 are based on the total concentration of *threo*-D_s-isocitrate. Under the conditions of these assays, *threo*-D_s-isocitrate will be present in varying amounts as the unprotonated and protonated forms and complexed with Mg^{2+} and Ca^{2+}. It is very unlikely that all these forms will be substrates for the enzyme. At concentrations of ADP of 2mM and above there was inhibition of enzyme activity, but no loss of the activation by Ca^{2+} (Fig. 3). The inhibition at high concentrations of ADP is probably the result of the binding of Mg^{2+} to ADP. Under the conditions used in Fig. 3, the addition of 2mM- and 5mM-ADP lowers Mg^{2+} concentration to about 400 and 200μM respectively in the presence of both 5mM-EGTA and 5mM-EGTA plus 5mM-$CaCl_2$.

The inhibitory effects of 5mM-EGTA in the presence of ADP and 0.1mM-*threo*-D_s-isocitrate could be completely reversed by the subsequent addition of either further *threo*-D_s-isocitrate (to give

Table 2. *Kinetic constants for rat heart NAD-ICDH with various concentrations of threo-*D_s*-isocitrate in the presence and absence of ADP and* Ca^{2+} *ions*

Data are taken from Fig. 4 and fitted to $v = V/\{1+(K_m/[S])^n\}$. Values ± S.D. (for 10–12 observations) are given in each case.

Additions	Calculated range of $[Ca^{2+}]$ (μM)	V (munits/mg of mitochondrial protein)	K_m (μM)	n
EGTA (5 mM)	<0.001	58.2 ± 2.34	280 ± 13	2.7 ± 0.32
EGTA (5 mM), $CaCl_2$ (5 mM)	33–29	57.3 ± 3.00	352 ± 26	2.4 ± 0.34
ADP (1 mM), EGTA (5 mM)	<0.001	60.0 ± 3.54	227 ± 17	2.7 ± 0.50
ADP (1 mM), EGTA (5 mM), $CaCl_2$ (5 mM)	28–25	62.4 ± 1.41	53 ± 2.2	3.3 ± 0.44

a final concentration of 1 mM) or 5 mM-$CaCl_2$ (the pH of the assay buffer decreased under these conditions to about 6.7). In both cases, there appeared to be a short lag period (less than 1 min) before the new rate was established.

Sensitivity of rat heart NAD-ICDH to Ca^{2+}, Sr^{2+} *and* Mn^{2+}

The above findings indicate that the K_m for *threo-*D_s-isocitrate in the presence of ADP is greatly diminished by Ca^{2+} at about 25 μM. The sensitivity to Ca^{2+} at 1 mM-ADP and 0.1 mM-*threo-*D_s-isocitrate was explored by using CaEGTA buffer mixtures (see the Experimental section). The results of a typical experiment are shown in Fig. 5. The maximum stimulation by Ca^{2+} was approx. 5-fold, and the K_m for this stimulation was 1.19 ± 0.06 μM. In similar but separate experiments, values for the K_m of 1.39 ± 0.17 μM and 0.54 ± 0.04 μM were obtained; the latter result was in the presence of 0.2 mM-DL-isocitrate.

When CaEGTA buffer mixtures were replaced with SrEGTA buffer mixtures, it was evident that Sr^{2+} could mimic the effect of Ca^{2+}, although a higher range of concentrations was required. The calculated K_m for the data shown in Fig. 5 is 3.06 ± 0.33 μM, but the maximum activity reached was close to that observed with Ca^{2+}. In contrast, no evidence for any effect of Mn^{2+} could be obtained at concentrations up to about 2.5 μM. Unfortunately it is not possible to explore the effect of Mn^{2+} above this concentration, as addition of further $MnCl_2$ will displace endogenous Ca^{2+} from the EGTA.

An important question is whether the changes in activity can be related to interaction of the enzyme with low concentrations of Ca^{2+} and Sr^{2+}, or whether the effects are secondary to changes in the concentration of the free and bound forms of *threo-*D_s-isocitrate, ADP, EGTA and Mg^{2+}. We have computed the concentrations of these forms under some critical conditions and these are shown together with activity of the enzyme in Table 3. The conditions have been arranged into three groups giving approx. 20, 60 and 90–100% of the maximum activity of the enzyme. It is evident that the activation associated with increasing concentrations of Ca^{2+} and Sr^{2+} cannot be explained in terms of alterations in the concentration of Mg^{2+}, free EGTA, free *threo-*D_s-isocitrate, magnesium *threo-*D_s-isocitrate, free ADP or MgADP. For example, changes in the concentrations of these forms as great as those seen with added $CaCl_2$ or $SrCl_2$ are observed with added $MnCl_2$ without discernible activation of NAD$^+$-ICDH activity. It seems likely that the effects of Ca^{2+} and Sr^{2+} are the result of direct interaction of these ions with the enzyme. But, on present evidence, the possibility that activation is the result of an increase in the concentration of *threo-*D_s-isocitrate and/or ADP complexed to Ca^{2+} or Sr^{2+} cannot be ruled out. However, the concentrations of these complexes present under conditions of half-maximal velocity (about 0.02 and 0.5 μM respectively) are low. Moreover, much higher concentrations of Mn^{2+} and Mg^{2+} complexes with isocitrate and ADP clearly have no effect.

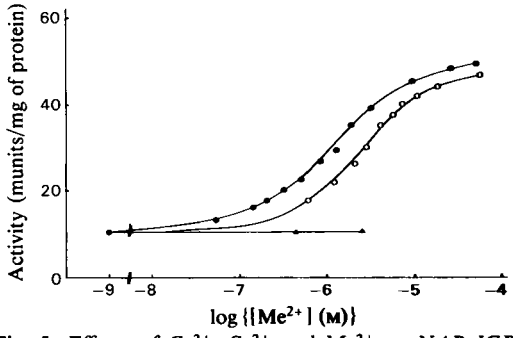

Fig. 5. *Effects of* Ca^{2+}, Sr^{2+} *and* Mn^{2+} *on NAD-ICDH activity*

Activity was assayed with 5 mM-EGTA and additions of $CaCl_2$ (●), $SrCl_2$ (○) and $MnCl_2$ (▲) to give the indicated concentration of the appropriate metal ion in the presence of 0.1 mM-*threo-*D_s-isocitrate, 2 mM-NAD^+, 1 mM-ADP and 1 mM-$MgCl_2$.

Effect of Ca^{2+} *ions on the activity of NAD-ICDH in extracts of mitochondria isolated from various tissues of the rat*

Results obtained with extracts prepared from heart, liver and kidney as well as brown and white

Table 3. *Relationship of the changes in NAD-ICDH activity with addition of $CaCl_2$, $SrCl_2$ and $MnCl_2$ in the presence of EGTA to changes in the concentrations of free and complexed forms of Mg^{2+}, EGTA, threo-D_s-isocitrate and ADP*

The concentrations of the various forms have been calculated as described in the Experimental section. Values of NAD-ICDH activity are taken from Fig. 5; details of assay conditions are given in the legend to that Figure. Me represents Ca, Sr or Mn as appropriate; calculated concentrations of free and complexed forms of *threo*-D_s-isocitrate are the sum of protonated and unprotonated species.

		Calculated concentrations in assay (μM)								
					threo-D_s-Isocitrate			ADP		
Additions to assay	NAD-ICDH activity (% of maximum)	Free Mg^{2+}	Free Me^{2+}	Free EGTA	Free	Mg complex	Me complex	Free	Mg complex	Me complex
None	20	543	0.001	4.89	79	21	—	673	327	—
5.0mM-$MnCl_2$	20	624	2.54	0.02	76	24	0.21	630	352	18
4.3mM-$CaCl_2$	59	608	1.30	0.68	77	23	0.03	648	352	0.49
0.2mM-$SrCl_2$	62	546	2.68	4.70	79	21	0.02	672	328	0.50
5.0mM-$CaCl_2$	100	622	27.5	0.04	76	24	0.59	636	353	10
5.0mM-$SrCl_2$	98	631	498	0.37	73	23	3.64	587	331	82
1.0mM-$SrCl_2$	90	557	16.0	3.93	78	22	0.21	666	331	2.98

Table 4. *Effects of Ca^{2+} ions on the activity of NAD-ICDH in extracts of mitochondria isolated from various tissues of the rat*

Mitochondria and extracts were prepared as given in the Experimental section. Samples were assayed for NAD-ICDH activity in the presence of 1mM-$MgCl_2$, 2mM-NAD^+ and 1mM-ADP at either 0.1 or 1mM-*threo*-D_s-isocitrate with either endogenous Ca^{2+} or 5mM-EGTA (Ca^{2+} concentration <1nM) or 5mM-EGTA and 5mM-$CaCl_2$ (Ca^{2+} concentration 27.5μM). Results are means of duplicate observations on two separate mitochondrial extracts (values agreed within 10%).

		Rates (munits/mg of mitochondrial protein)					
	threo-D_s-Isocitrate concn. ...	0.1mM			1mM		
Source of mitochondria	[Ca^{2+}] ...	Endogenous	1nM	27.5μM	Endogenous	1nM	27.5μM
Heart		53	6.5	52	53	50	52
Liver		10.0	2.1	8.1	7.5	5.8	5.8
Kidney		13	1.1	13	17	15	16
Brown interscapular adipose tissue		102	6.8	82	100	95	91
White epididymal adipose tissue		42	4.2	40	42	39	42

adipose tissue are shown in Table 4. Activities in all cases were measured with ADP at both 0.1mM- and 1mM-*threo*-D_s-isocitrate in the presence of either endogenous Ca^{2+}, 5mM-EGTA (i.e. Ca^{2+} concentration <1nM) or 5mM-EGTA plus 5mM-$CaCl_2$ (i.e. Ca^{2+} concentration about 27.5μM).

Although there were marked differences in the maximum activity of NAD-ICDH observed when expressed in terms of mitochondrial protein, the pattern of changes in activities was very similar. Alterations in Ca^{2+} concentration had little effect on activity in the presence of 1mM-*threo*-D_s-isocitrate, but at 0.1mM-*threo*-D_s-isocitrate there was a 4–12-fold difference in activity between high and low Ca^{2+} concentrations. The sensitivity to Ca^{2+} was determined as described in Fig. 5 by using extracts of mitochondria from kidney and epididymal white adipose tissue. The K_m values for Ca^{2+} were 1.67±0.32 and 1.02±0.13μM respectively. These values are very close to that for the heart enzyme (Fig. 5). It would appear that activation by Ca^{2+} in the presence of ADP may be a common feature of NAD-ICDH from mammalian sources. Zammit & Newsholme (1976) had concluded from their studies that only NAD-ICDH from contractile tissues was sensitive to Ca^{2+}.

Ca^{2+} concentration and the regulation of NADP-ICDH activity in extracts of rat heart mitochondria

As found by others (see Colman, 1975), the maximum activity of NADP-ICDH in extracts of rat heart mitochondria was some 10-fold greater than that of NAD-ICDH. No effects of EGTA or varying Ca^{2+} concentration were observed.

General conclusions

Extensive studies of the kinetic behaviour of NAD-ICDH from mammalian hearts have been made in the absence of EGTA (Plaut *et al.*, 1974; Cohen & Colman, 1974; Siliski & Colman, 1974). Without special precautions it is likely that the concentration of Ca^{2+} was saturating in these previous investigations and thus a substantial lowering of the apparent K_m for *threo*-D_s-isocitrate with ADP was generally observed. However, in certain circumstances, for example in the presence of high concentrations of ADP, it is possible, even in the absence of EGTA, that the concentration of Ca^{2+} may become limiting. The heart enzyme appears to have an enzymically active protomer of mol.wt. 330000 that associates in the presence of ADP to dimeric and tetrameric forms (Giorgio *et al.*, 1970; Shen *et al.*, 1974). The dependency of this association on the concentration of Ca^{2+} remains to be explored.

It is always risky to ascribe regulatory significance to enzyme properties when the evidence is restricted to findings with the separated enzyme, as in this present study. This is certainly so with NAD-ICDH, since it has not been demonstrated that the step catalysed by the enzyme is out of equilibrium in whole-cell or mitochondria preparations from mammalian tissues. Moreover, the maximum activity of NADP-ICDH is an order of magnitude greater than that of NAD-ICDH. However, there is evidence to suggest that the transfer of reducing power from NADPH to the respiratory chain is slow compared with that from NADH in mitochondria from mammalian sources (Nicholls & Garland, 1969; Plaut & Smith, 1977). The high activity of NADP-ICDH in the rat heart may explain the substantial rates of isotopic exchange that were observed between [citrate+isocitrate] and both HCO_3^- and 2-oxoglutarate in a previous study using the perfused rat heart (Randle *et al.*, 1970).

An interesting inference that can be drawn from the sensitivity of NAD-ICDH to Ca^{2+} is that the intramitochondrial Ca^{2+} concentrations may be of the order of 1 μM, that is, no more than 10–100 times the concentration in the cytoplasm. The cytoplasmic concentration of Ca^{2+} has been measured fairly directly in a few large muscle and nerve cells, but the concentration in the cytoplasm of other cells has been inferred largely from the Ca^{2+}-sensitivity of regulatory proteins, which respond to changes in cytoplasmic Ca^{2+}, such as phosphorylase *b* kinase, tropomyosin and the FAD-linked glycerol phosphate dehydrogenase. There are at least three exclusively intramitochondrial enzymes (pyruvate dehydrogenase phosphate phosphatase, pyruvate dehydrogenase kinase and NAD-ICDH) that are sensitive to changes in Ca^{2+} at concentrations of approx. 1 μM, and thus it is not unreasonable to take this as an estimate of the concentration of Ca^{2+} in mitochondria. If this estimate is correct, it is incompatible with the distribution of Ca^{2+} across the mitochondrial inner membrane being determined solely by the membrane potential (for further discussion of this point, see: Crompton *et al.*, 1976, 1977, 1978; Denton, 1977; Pozzan *et al.*, 1977).

It is an intriguing possibility that an increase in the intramitochondrial concentration of Ca^{2+} may be a common mechanism for enhancing the rates of utilization of a number of respiratory fuels. In addition to the direct effects that have been demonstrated on the interconverting enzymes of the pyruvate dehydrogenase system (Denton *et al.*, 1972; Cooper *et al.*, 1974) and NAD–isocitrate dehydrogenase (the present paper), evidence has been presented that increases in intramitochondrial Ca^{2+} concentration may enhance the rate of oxidation of β-hydroxybutyrate (Malmström & Carafoli, 1976), succinate (Ezawa & Ogata, 1977) and fatty acids (Otto & Ontko, 1978).

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