Characterization of the effects of Ca^{2+} on the intramitochondrial Ca^{2+} -sensitive enzymes from rat liver and within intact rat liver mitochondria

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1. The regulatory properties of the Ca^{2+} -sensitive intramitochondrial enzymes (pyruvate dehydrogenase phosphate phosphatase, NAD^+ -isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase) in extracts of rat liver mitochondria appeared to be essentially similar to those described previously for other mammalian tissues. In particular, the enzymes were activated severalfold by Ca^{2+} , with half-maximal effects at about $1\ \mu\text{M}$ - Ca^{2+} ($K_{0.5}$ value). 2. In intact rat liver mitochondria incubated in a KCl-based medium containing 2-oxoglutarate and malate, the amount of active, non-phosphorylated, pyruvate dehydrogenase could be increased severalfold by increasing extramitochondrial $[\text{Ca}^{2+}]$, provided that some degree of inhibition of pyruvate dehydrogenase kinase (e.g. by pyruvate) was achieved. 3. The rates of $^{14}\text{CO}_2$ production from 2-oxo-[1- ^{14}C]glutarate at non-saturating, but not at saturating, concentrations of 2-oxoglutarate by the liver mitochondria (incubated without ADP) were similarly enhanced by increasing extramitochondrial $[\text{Ca}^{2+}]$. 4. The rates and extents of NAD(P)H formation in the liver mitochondria induced by non-saturating concentrations of 2-oxoglutarate, glutamate, *threo*-D_s-isocitrate or citrate were also increased in a similar manner by Ca^{2+} under several different incubation conditions, including an apparent 'State 3.5' respiration condition. Ca^{2+} had no effect on NAD(P)H formation induced by β -hydroxybutyrate or malate. 5. In intact, fully coupled, rat liver mitochondria incubated with 10 mM-NaCl and 1 mM-MgCl₂, the apparent $K_{0.5}$ values for extramitochondrial Ca^{2+} were about $0.5\ \mu\text{M}$, and the effective concentrations were within the expected physiological range, 0.05 – $5\ \mu\text{M}$. In the absence of Na^+ , Mg^{2+} or both, the $K_{0.5}$ values were about 400, 200 and 100 nM respectively. These effects of increasing extramitochondrial $[\text{Ca}^{2+}]$ were all inhibited by Ruthenium Red. 6. When extramitochondrial $[\text{Ca}^{2+}]$ was increased above the effective ranges for the enzymes, a time-dependent deterioration of mitochondrial function and ATP content was observed. 7. The implications of these results on the role of the Ca^{2+} -transport system of the liver mitochondrial inner membrane are discussed.

INTRODUCTION

In mammalian tissues there are three exclusively intramitochondrial dehydrogenases that occupy key regulatory sites in oxidative metabolism which can be activated severalfold by increasing the concentration of free Ca^{2+} ions within the range 0.1 – $10\ \mu\text{M}$, with $K_{0.5}$ values for Ca^{2+} of about $1\ \mu\text{M}$. They are PDH, NAD-ICDH and OGDH (for reviews see Denton & McCormack, 1980, 1981; Hansford, 1985). Ca^{2+} activates PDH indirectly by causing increases in the amount of PDH_a through its activation of PDHP-phosphatase (Denton *et al.*, 1972), and perhaps also through inhibition of PDH_a kinase (Cooper *et al.*, 1974). NAD-ICDH and OGDH are activated more directly by Ca^{2+} , which causes decreases in their K_m values for their respective substrates, *threo*-D_s-isocitrate (Denton *et al.*, 1978) and 2-oxoglutarate (McCormack & Denton, 1979).

The regulation of these enzymes by Ca^{2+} has been

demonstrated in extracts from all vertebrate tissues so far examined, but appears not to be evident in extracts of invertebrates (see Denton & McCormack, 1980; McCormack & Denton, 1981). Although they have been stated to be Ca^{2+} -sensitive (see Denton & McCormack, 1980), results on the effects of Ca^{2+} on these enzymes from mammalian liver have not yet been reported. The present paper includes detailed kinetic analyses of the effects of Ca^{2+} on all three of these Ca^{2+} -sensitive intramitochondrial enzymes in extracts prepared from rat liver mitochondria.

Studies on intact, fully coupled, mitochondria from a variety of different mammalian tissues (see the Results and discussion section) have demonstrated that these Ca^{2+} -sensitive intramitochondrial dehydrogenases can be activated by increases in the extramitochondrial concentration of Ca^{2+} within the expected physiological range (i.e. about 0.05 – $5\ \mu\text{M}$; see Rasmussen & Barrett, 1984), with $K_{0.5}$ values for Ca^{2+} of about $0.5\ \mu\text{M}$. This is achieved

Abbreviations used: PDH, pyruvate dehydrogenase complex; PDH_a, the active, non-phosphorylated, form of the pyruvate dehydrogenase complex; PDHP-phosphatase, pyruvate dehydrogenase phosphate phosphatase; NAD-ICDH, NAD⁺-linked isocitrate dehydrogenase (EC 1.1.1.41); OGDH, the 2-oxoglutarate dehydrogenase complex; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine; $K_{0.5}$ value, the concentration of effector required for half-maximal response; HEDTA, N-hydroxyethylethylenediaminetriacetic acid.

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by incubation in the presence of physiological extra-mitochondrial concentrations of Mg^{2+} and Na^+ , which inhibit Ca^{2+} uptake by, and promote Ca^{2+} egress from, mitochondria, respectively (see Carafoli, 1979; Nicholls & Crompton, 1980). In the absence of either or both of these ions, the observed $K_{0.5}$ values for extramitochondrial Ca^{2+} are (additively) decreased (see Denton & McCormack, 1980) by magnitudes dependent on the magnitudes of their respective effects on Ca^{2+} transport by the mitochondria under study. Thus in this way the Ca^{2+} -transport systems of the mitochondrial inner membrane can be studied in the presence of physiological concentrations of extramitochondrial Ca^{2+} and under conditions of apparently physiological Ca^{2+} loading of the mitochondria.

More importantly, however, these previous studies on intact mammalian mitochondria led to the proposal that hormones and other agents which cause increases in cytoplasmic $[Ca^{2+}]$ may thereby cause increases in intramitochondrial $[Ca^{2+}]$ and so bring about stimulations of mitochondrial oxidative metabolism through the activation of these key Ca^{2+} -sensitive dehydrogenases (Denton & McCormack, 1980, 1981). The ability to assay the Ca^{2+} -sensitive properties of these enzymes within intact rat heart mitochondria has allowed strong evidence in support of this view to be obtained in studies on the β -adrenergic-agonist activation of rat heart PDH and OGDH (McCormack & Denton, 1984).

The previous studies mentioned in the two preceding paragraphs relied on relatively simple and straightforward methods for assaying the Ca^{2+} -sensitive properties of these enzymes within intact mitochondria. However, preliminary work revealed that the application of these techniques (as used previously) failed to demonstrate the Ca^{2+} -sensitivity of these enzymes when they were located within intact liver mitochondria (see the Results and discussion section). Moreover, other workers had proposed that the concentration of Ca^{2+} within liver mitochondria in physiological situations would always be above the range in which the enzymes show regulation by Ca^{2+} (see, e.g., Williamson *et al.*, 1981; Joseph *et al.*, 1983). The present paper, however, shows that it is most likely that the regulation of these enzymes by intramitochondrial Ca^{2+} ions in liver has physiological relevance, and describes techniques and conditions whereby the Ca^{2+} -sensitive properties of these enzymes can be studied within intact rat liver mitochondria. The following paper (McCormack, 1985b) describes studies on the utilization of these techniques to obtain evidence that hormones which cause increases in liver cytoplasmic $[Ca^{2+}]$ also cause increases in intramitochondrial $[Ca^{2+}]$ and thus activation of the Ca^{2+} -sensitive intramitochondrial dehydrogenases. Results similar to a small part of the present work have been published previously in a short article (McCormack, 1985a).

EXPERIMENTAL

Preparation of mitochondria

Mitochondria were prepared from the livers of decapitated male or female (as indicated in the Results and discussion section) fed Wistar rats of 225–275 g body wt., by the method described by McCormack (1985a); the weight of the animals, within this range, did not affect the results obtained, and essentially similar results could also be obtained if anaesthetized rats were

used (see McCormack, 1985a). This procedure involved the disruption of the liver with a Polytron homogenizer [two or three short periods (2–3 s) at mark 4] in a sucrose-based medium (5 ml/g of tissue) containing 2 mM-EGTA and 1% (w/v) albumin, and then included a Percoll purification step based on the method of Belsham *et al.* (1980) as adapted by McCormack (1985a). This step was particularly important for improving the signal:noise ratio in the experiments in which a dual-wavelength spectrophotometer was used (see below), and resulted in a 1.5–2-fold purification of the mitochondria when assessed on the basis of total PDH activity/mg of protein, with little effect on the yield of total PDH activity. However, it should be noted that essentially similar results could be obtained with mitochondria that had not been subjected to this step, except that about twice as much sample protein had to be added to obtain the same values as those presented in the Results and discussion section (results not shown). It should also be noted that the mitochondria obtained appeared to be well coupled (results not shown; Chance & Williams, 1956), and that results essentially similar to those presented below could be obtained with mitochondria that were prepared in mannitol-based media rather than a sucrose-based one (results not shown); there has been a considerable controversy over which is the better supporting medium to use when preparing liver mitochondria (see, e.g., Whipps & Halestrap, 1984).

Extraction of mitochondria and assays of enzymes

The extraction of mitochondria for the assay of PDHP-phosphatase activity was as described by McCormack & Denton (1980). This enzyme was assayed by incubating extracts (for various times at 30 °C in buffer containing 25 mM- $MgCl_2$) as described by McCormack & Denton (1980) and monitoring the rates of increase in the amounts of PDH_a derived from endogenous PDH phosphate (at different concentrations of Ca^{2+} or Sr^{2+}) as described by Marshall *et al.* (1984). $K_{0.5}$ values for Ca^{2+} and Sr^{2+} were calculated both from the initial rates of increase in the amount of PDH_a achieved at the various concentrations of Ca^{2+} or Sr^{2+} , and from the actual values of PDH_a (minus the initial, i.e. zero-time, value of PDH_a) at given time-points, provided that at saturating $[Ca^{2+}]$ or $[Sr^{2+}]$ the amount of PDH_a was still below the total amount of PDH present.

Mitochondria were extracted and assayed for NAD-ICDH activity as described by Denton *et al.* (1978). OGDH was extracted, and then partially purified, before being assayed as described by McCormack & Denton (1979). A unit of enzyme activity is defined as the amount that catalyses the conversion of 1 μ mol of substrate/min at 30 °C.

Incubation of mitochondria and techniques used to assay the intramitochondrial Ca^{2+} -sensitive dehydrogenases within intact liver mitochondria

Mitochondria were incubated at 0.5–1.5 mg of protein/ml and 30 °C in a basic medium consisting of 125 mM-KCl, 20 mM-Tris (pH 7.3) (or 20 mM-Mops when at pH 7.0 or 7.1) and 5 mM-potassium phosphate, except that when 10 mM-NaCl was present (see below) only 115 mM-KCl was used. Additions or changes to this medium are indicated in the Figures and Tables. Potassium salts were used throughout unless otherwise stated.

Table 1. Summary of the kinetic properties of the Ca^{2+} -sensitive intramitochondrial enzymes from rat liver

Details for the preparation of extracts, the partial purification of OGDH, the assay of the enzymes, and the calculations of kinetic parameters and free Ca^{2+} concentrations are given in the Experimental section. Assays [and the prior incubations for (a)] were at pH 7 and 30 °C except where indicated, and other additions to the appropriate assay buffers (see the Experimental section) were as shown. Results are given as either (i–iii) $K_{0.5}$ or K_m values $\pm$ s.d. for the numbers of degrees of freedom in parentheses, based on observations made on samples from at least three different preparations of mitochondria, or (iv) as means $\pm$ s.e.m. for the numbers of different mitochondrial preparations in parentheses (the V_{max} activity of OGDH was assayed at a suitable saturating concentration of 2-oxoglutarate). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ versus the appropriate control value.

Enzyme	Parameter (units)	Other conditions	Parameter value
(a) PDHP-phosphatase	(i) $K_{0.5}$ for Ca^{2+} (μM)	—	1.41 ± 0.32 (18)
	(ii) $K_{0.5}$ for Sr^{2+} (μM)	—	16.1 ± 2.4 (18)
(b) NAD-ICDH	(i) $K_{0.5}$ for Ca^{2+} (μM)	At 0.1 mM-threo- D_5 -isocitrate	0.91 ± 0.21 (22)
	(ii) $K_{0.5}$ for Sr^{2+} (μM)	At 0.1 mM-threo- D_5 -isocitrate	10.4 ± 1.7 (17)
	(iii) K_m for threo- D_5 -isocitrate (mM)	At $[\text{Ca}^{2+}] < 1 \text{ nM}$	0.39 ± 0.05 (27)
		At $[\text{Ca}^{2+}] \approx 30 \mu\text{M}$	0.08 ± 0.01 (27)
(c) OGDH	(i) $K_{0.5}$ for Ca^{2+} (μM)	At 0.1 mM-2-oxoglutarate	1.22 ± 0.10 (24)
		At 0.05 mM-2-oxoglutarate plus 1 mM-ADP	0.41 ± 0.05 (17)
	(ii) $K_{0.5}$ for Sr^{2+} (μM)	At 0.1 mM-2-oxoglutarate	11.4 ± 0.9 (18)
	(iii) K_m for 2-oxoglutarate (mM)	At $[\text{Ca}^{2+}] < 1 \text{ nM}$	2.41 ± 0.31 (26)
		At $[\text{Ca}^{2+}] \approx 30 \mu\text{M}$	0.21 ± 0.03 (25)
	(iv) Activity at the given non-saturating [2-oxoglutarate] (as % of the V_{max} activity)	(1) At $[\text{Ca}^{2+}] < 1 \text{ nM}$ and 2 mM-2-oxoglutarate and:	
		No further additions (control)	41.3 ± 3.2 (6)
		1 mM-ADP	82.4 ± 6.0 (4)***
		1 mM-ATP	18.3 ± 2.4 (3)**
		At pH 7.2	24.9 ± 2.5 (3)*
		(2) At $[\text{Ca}^{2+}] \approx 30 \mu\text{M}$ and 0.1 mM-2-oxoglutarate and:	
		No further additions (control)	37.4 ± 2.9 (5)
		1 mM-ADP	77.8 ± 5.2 (4)***
		1 mM-ATP	24.1 ± 1.9 (3)**
		At pH 7.2	26.3 ± 2.0 (3)*

Samples (1 ml) in which PDH activity was to be assayed were rapidly sedimented (10000 *g* for 20 s) and quickly frozen in liquid N_2 before extraction and assay for both the amount of PDH_a and the total amount of PDH present, as described by McCormack *et al.* (1982). Results are generally given as the percentage of total enzyme existing as PDH_a.

OGDH activity was assayed in intact liver mitochondria by measuring either $^{14}\text{CO}_2$ production from 2-oxo-[1- ^{14}C]glutarate as described by McCormack (1985a), or 2-oxoglutarate-dependent NAD(P)⁺ reduction by using a dual-wavelength spectrophotometer as described by McCormack *et al.* (1982); the wavelength pair used was 350–370 nm. NAD-ICDH activity in the intact mitochondria was also monitored with the dual-wavelength spectrophotometer, except that threo- D_5 -isocitrate was the substrate.

Use of Ca-EGTA buffers, assays of mitochondrial protein, ATP and O_2 uptake, and methods of kinetic and statistical analyses

Ca-EGTA, Ca-HEDTA and Sr-EGTA buffers were prepared and used (at 5 mM ligand unless otherwise stated), and free metal ion concentrations were calculated, as described by Denton *et al.* (1978) and McCormack *et al.* (1982). These calculations made direct use of stability constants obtained from Martell & Smith (1974), although it is appreciated that this may not yield the absolutely correct concentrations of metal ion, as

allowances for the differences between these constants and the chemical activity of both protons and the metal ions were not made (see, e.g., Tsien & Rink, 1980; Miller & Smith, 1984); however, there is still considerable controversy over what is the correct procedure for these calculations (see, e.g., Tsien & Rink, 1980; Miller & Smith, 1984).

Mitochondrial protein was measured by the method of Gornall *et al.* (1949), and mitochondrial ATP content by the method of Stanley & Williams (1969), in samples prepared as described by McCormack & Denton (1980). Oxygen uptake was measured with a Clark-type oxygen electrode as described by McCormack & Denton (1980). Kinetic constants were calculated by fitting data to equations of the type:

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

by using a non-linear least-squares regression programme written for a Hewlett-Packard 8945 computer by Dr. Paul J. England (Smith, Kline and French Research, Welwyn, Herts., U.K.). Note that the K_m or $K_{0.5}$ values given were based on observations made over suitable ranges of substrate or free Ca^{2+} (or Sr^{2+}) concentrations, as appropriate. Statistical significance was assessed by Student's *t* test.

Chemicals, biochemicals and radiochemicals

All chemicals and biochemicals were obtained from Boehringer Corp., Lewes, East Sussex, U.K., or BDH

Chemicals, Poole, Dorset, U.K., or Sigma, Poole, Dorset, U.K., except: rotenone (Aldrich Chemical Co., Gillingham, Dorset, U.K.); diltiazem {*cis*-(+)-3-acetyloxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one}, which was kindly given by Dr. G. Satzinger, of Goedecke A.G., 78 Freiburg, Germany; and ionophore A23187, which was kindly given by Dr. R. L. Hamill, of Eli Lilly and Co., Indianapolis, IN, U.S.A. 2-Oxo-[1-¹⁴C]glutarate and [1-¹⁴C]glutamate were obtained from Amersham International, Amersham, Bucks., U.K.

RESULTS AND DISCUSSION

Effects of Ca^{2+} , and other effectors, on the activities of the Ca^{2+} -sensitive intramitochondrial enzymes in extracts of rat liver mitochondria

In extracts of mitochondria which had undergone the Percoll purification step (see the Experimental section), the total activity of PDH and the $V_{\max}$ activities (i.e. assayed at saturating [substrate]) of NAD-ICDH and OGDH (as munits/mg of protein) were 44.4 ± 2.3 (40), 38.2 ± 3.8 (12) and 35.4 ± 5.8 (6) respectively; values are means $\pm$ S.E.M. for the numbers of different mitochondrial preparations in parentheses. These values, in each case, represent a 1.5–2-fold purification of activity (with respect to protein) as the result of this purification step. However, they are still only about one-third of the corresponding values for heart (Denton *et al.*, 1980) or brown- or white-adipose-tissue (McCormack & Denton, 1980; Marshall *et al.*, 1984) mitochondria. This may indicate that the preparations used in the present study are still very contaminated by other cellular fractions, or that these enzymes constitute a lesser proportion of total protein in liver mitochondria than in these other mitochondria. None of the above $V_{\max}$ or total values was altered by the treatments described below.

The data in Table 1 show that the regulatory properties of PDHP-phosphatase, NAD-ICDH and OGDH that were exhibited in extracts of rat liver mitochondria were essentially similar to those exhibited by these enzymes in extracts of mitochondria prepared from rat heart and both brown and white adipose tissue (Denton *et al.*, 1980; McCormack & Denton, 1980; Marshall *et al.*, 1984). In particular, each enzyme was activated by Ca^{2+} in the range 0.1–10 μM , with $K_{0.5}$ values of around 1 μM . It should be noted that Ca^{2+} could stimulate activity by up to at least 4-fold in each case (results not shown in full; Table 1). As has also been shown in mitochondrial extracts from the other tissues just mentioned (see references just cited), the liver enzymes could also be activated by Sr^{2+} , but at approx. 10-fold higher concentrations than for Ca^{2+} (Table 1).

An early study on PDHP-phosphatase (Denton *et al.*, 1972) had suggested that this enzyme from liver may be less sensitive to activation by Ca^{2+} than that from other tissues. However, this was probably due to proteinase activity (Wieland, 1975; Lynen *et al.*, 1978) in the mitochondrial extracts. This problem was overcome in the present study by extracting mitochondria in the presence of rat serum (see the Experimental section and also Hems *et al.*, 1978). In a previous study, Roche & Cate (1977) briefly mentioned that pig liver PDHP-phosphatase could be activated by Ca^{2+} , with a $K_{0.5}$ value in the range 1–3 μM .

Fig. 1. Effects of Ca^{2+} on the amount of PDH_a in intact rat liver mitochondria isolated from both male and female rats and incubated in the presence of different concentrations of pyruvate

Mitochondria prepared from either male (open symbols, broken lines) or female (closed symbols, continuous lines) rat livers were incubated for 5 min, at 30 °C and pH 7.3 and approx. 1 mg of protein/ml, in 1 ml of KCl-based medium (see the Experimental section) containing 2 mM-2-oxoglutarate and 0.2 mM-L-malate together with appropriate amounts of EGTA-Ca buffers (at 5 mM-EGTA) to give the concentrations of Ca^{2+} shown, and the following other additions: (▼, ▽) none, (▲, △) 0.25 mM-, (●) 0.5 mM- or (■, □) 1 mM-pyruvate. The total activity of PDH was unaffected by the treatments used (see the Experimental section). Results shown are the means of values obtained from at least three different preparations of mitochondria; S.E.M. values for these were all within 15% of the values shown.

For NAD-ICDH activity, the extract was prepared and assayed in the presence of 1 mM-ADP. In the absence of ADP, Ca^{2+} no longer activated the liver enzyme (results not shown), as has been found previously for the enzyme from other tissues (see Denton *et al.*, 1978). The K_m value of rat liver NAD-ICDH for *threo*-D₅-isocitrate was also markedly increased by a modest increase in pH (results not shown, but similar to those shown for OGDH in Table 1), as has been found previously for the pig heart enzyme (Colman, 1975).

OGDH was partially purified before being assayed (see the Experimental section), as its activity was the most intensively studied in the present paper (see below), and a convenient method existed. The mean ($\pm$ S.E.M.) activity of OGDH in three such preparations was 3.1 ± 0.3 units/mg of protein. Table 1 shows that the K_m of rat liver OGDH could also be increased by an increase in pH or by ATP, or be decreased by ADP, as has been found

Table 2. Kinetic parameters for the effects of Ca^{2+} on the PDH_a content of intact rat liver mitochondria incubated under a variety of different conditions

Details of the method of calculation are given in the Experimental section. The incubation conditions were as in the legend to Fig. 1 together with additions or changes as shown. Results are given as means (PDH_a values) or $K_{0.5}$ values as described for Table 1.

Sex of rat	Basic incubation conditions	Other additions or changes	PDH _a content of mitochondria incubated with:		
			EGTA alone	the maximally effective $[\text{Ca}^{2+}]$	$K_{0.5}$ value for extramitochondrial Ca^{2+} (nM)
Male	2 mM-2-Oxoglutarate + 0.2 mM-L-malate	0.5 mM-Pyruvate	17 ± 4 (3)	60 ± 8 (3)	109 ± 16 (18)
		1 mM-Pyruvate	25 ± 3 (4)	81 ± 8 (4)	89 ± 11 (23)
Female	2 mM-2-Oxoglutarate + 0.2 mM-L-malate	0.5 mM-Pyruvate	13 ± 4 (5)	32 ± 5 (4)	110 ± 10 (39)
		1 mM-Pyruvate	18 ± 2 (8)	59 ± 4 (7)	98 ± 9 (45)
		1 mM-Dichloroacetate	15 ± 4 (3)	39 ± 5 (3)	84 ± 12 (17)
	2 mM-2-Oxoglutarate + 0.2 mM-L-malate + 1 mM-pyruvate	Oligomycin (5 µg/ml) with:			
		0.25 mM-ADP	8 ± 1 (3)	20 ± 3 (3)	120 ± 15 (18)
		1 mM-ADP	19 ± 3 (4)	51 ± 6 (3)	96 ± 5 (21)
		0.25 mM-ADP + 0.5 mM-pyruvate	26 ± 4 (3)	79 ± 7 (3)	84 ± 10 (18)
		10 mM-NaCl	19 ± 3 (4)	60 ± 6 (4)	184 ± 21 (26)
		1 mM-MgCl ₂	19 ± 2 (4)	59 ± 8 (4)	356 ± 43 (28)
		10 mM-NaCl + 1 mM-MgCl ₂	18 ± 2 (6)	63 ± 4 (6)	484 ± 29 (43)
		At pH 7	13 ± 1 (3)	48 ± 4 (3)	101 ± 12 (21)
		At pH 7, with 10 mM-NaCl + 1 mM-MgCl ₂	13 ± 3 (3)	45 ± 5 (3)	462 ± 39 (27)
		1 mM-Dichloroacetate	14 ± 2 (4)	40 ± 6 (4)	1059 ± 10 (25)
		1 µM-FCCP, 2 mM-MgATP, 4 mM-MgCl ₂ , oligomycin (5 µg/ml), A23187 (2 µg/ml), 1 µM-valinomycin			

previously for the pig heart (McCormack & Denton, 1979), rat heart (Denton *et al.*, 1980) and bovine kidney (Lawlis & Roche, 1981) enzymes. ADP also caused a decrease in the $K_{0.5}$ value for the activation of liver OGDH by Ca^{2+} (Table 1), as was previously noted with rat heart OGDH (Denton *et al.*, 1980).

Activation of PDH in intact rat liver mitochondria by increases in the extramitochondrial concentration of Ca^{2+}

When intact mitochondria isolated from rat heart (Denton *et al.*, 1980; Hansford, 1981), skeletal muscle (Ashour & Hansford, 1983; Fuller & Randle, 1984), epididymal adipose tissue (Marshall *et al.*, 1984) or brain (Hansford & Castro, 1985), or within permeabilized pig lymphocytes (Baumgarten *et al.*, 1983), are incubated in KCl-based medium containing phosphate and respiratory substrates (e.g. 2-oxoglutarate with malate), the effects of intramitochondrial Ca^{2+} on PDHP-phosphatase can be assayed indirectly by measuring increases in steady-state PDH_a content as the extramitochondrial $[\text{Ca}^{2+}]$ is increased. This is because PDH_a kinase activity is likely to be constant under these conditions (see McCormack & Denton, 1980; Denton *et al.*, 1980). With intact rat liver mitochondria (Fig. 1) it was found to be also necessary to add some pyruvate, or its analogue dichloroacetate, or some ADP with oligomycin (Table 2), to inhibit PDH_a kinase to some degree (see Whitehouse *et al.*, 1974;

Denton *et al.*, 1975; McCormack *et al.*, 1982), so that the effects of Ca^{2+} on the phosphatase could be observed in this manner. Otherwise PDH_a values were very low and the effects of Ca^{2+} small and difficult to observe (Fig. 1). This is somewhat analogous to the situation with heart mitochondria prepared from starved or diabetic rats (see Kerbey *et al.*, 1976; McCormack *et al.*, 1982), and presumably means that, in the absence of these inhibitors of PDH_a kinase, its activity was far greater than that of the phosphatase under the conditions used (see Kerbey & Randle, 1981). The effects of pyruvate and ADP/oligomycin appeared to be additive (Table 2), as was also noted in studies on rat heart mitochondria (McCormack *et al.*, 1982).

The sex of the donor animal appeared to influence the degree of activation of PDH observed at any given concentration of pyruvate or Ca^{2+} (Fig. 1), though the $K_{0.5}$ values for Ca^{2+} were similar in each case (Table 2). No effects of sex on total PDH activity, mitochondrial ATP content, or OGDH activity within the mitochondria (see legend to Fig. 3) were observed. The reason(s) for the lower amounts of PDH_a in the mitochondria from the females is as yet unknown, but has also been noted in heart mitochondria (J. G. McCormack, unpublished work).

The addition of Na^+ and/or Mg^{2+} ions to the incubations of the liver mitochondria (in the presence of pyruvate) produced the expected increases in the $K_{0.5}$

values for Ca^{2+} (Table 2, Fig. 2). However, it is noticeable that the effects of Na^+ were much less, and that in the absence of Na^+ and Mg^{2+} the $K_{0.5}$ values were considerably higher, than those observed with rat heart (Denton *et al.*, 1980; Hansford, 1981), skeletal-muscle (Ashour & Hansford, 1983; Fuller & Randle, 1984) or epididymal-adipose-tissue (Marshall *et al.*, 1984) mitochondria. This is presumably because in liver mitochondria the Na^+ -dependent egress of Ca^{2+} is slower, and the Na^+ -independent egress faster, compared with these other types (see Carafoli, 1979; Nicholls & Crompton, 1980). The concentrations of Na^+ and Mg^{2+} used (Table 2) appeared to be saturating for their effects on the $K_{0.5}$ values for Ca^{2+} , as was also the case for the studies on OGDH (see below). It should also be noted that all of the effects of Na^+ reported in the present paper could be blocked by 300 μM -diltiazem (results not shown; but see McCormack, 1985a, b); this drug is known to be a potent inhibitor of the Na^+ -dependent egress of Ca^{2+} from both rabbit and rat heart mitochondria (Vághy *et al.*, 1982; McCormack & Denton, 1984).

A modest decrease in pH resulted in decreases in the amount of PDH_a at all pyruvate and Ca^{2+} concentrations (not shown in full, Table 2); however, the $K_{0.5}$ value for Ca^{2+} was unaffected. This has also been observed with mitochondria from rat heart and white adipose tissue (J. G. McCormack, unpublished work) and is in contrast with the activation of OGDH observed after a similar decrease in pH (see Table 3). This latter effect is probably due to the effects of pH on the enzyme itself (see Table 1); however, the mechanism(s) underlying the former effect are as yet unknown. However, as the activities of the two enzymes are affected in opposite directions by pH, it makes it very unlikely that any of the observed effects of Ca^{2+} are due to its causing changes in intramitochondrial pH.

All of the observed effects of increasing extramitochondrial $[\text{Ca}^{2+}]$ on PDH_a in coupled liver mitochondria could be inhibited by Ruthenium Red (not shown in full, Fig. 2), a potent inhibitor of mitochondrial Ca^{2+} uptake (Moore, 1971; Vasington *et al.*, 1972), thus providing strong evidence that they were due to increases in intramitochondrial $[\text{Ca}^{2+}]$.

The effects of Ca^{2+} on rat heart (Denton *et al.*, 1980) and white-adipose-tissue (McCormack & Denton, 1980) PDH_a -phosphatase can also be demonstrated in uncoupled (with FCCP) mitochondria incubated in the absence of respiratory substrates, but with ATPMg and oligomycin to allow constant kinase activity (see McCormack & Denton, 1980); in the latter case only it was also necessary to add the ionophore A23187 so that free Ca^{2+} equilibrium was achieved across the mitochondrial inner membrane. Table 2 shows that similar results could be obtained with uncoupled liver mitochondria, except that the addition of both of the ionophores A23187 and valinomycin was necessary to achieve free Ca^{2+} -equilibrium in this case, and once again some degree of PDH_a kinase inhibition (by dichloroacetate) was required.

Essentially similar results (not shown) to those presented in Fig. 1 and Table 2 could be obtained if: (i) incubations were at 25 or 35 $^{\circ}\text{C}$ instead of 30 $^{\circ}\text{C}$, (ii) the medium $[\text{KCl}]$ was either 100 or 150 mM instead of 125 mM, (iii) oxoglutarate with malate was replaced by either 2 mM-succinate or 5 mM-glutamate with 0.5 mM-L-malate, (iv) the required Ca^{2+} concentrations were

Fig. 2. Effects of increasing extramitochondrial $[\text{Ca}^{2+}]$ on (a) the ATP content of, and (b) the amount of PDH_a in, intact rat liver mitochondria incubated in the absence or presence of Na^+ and Mg^{2+} , or Ruthenium Red

Mitochondria prepared from the livers of female rats were incubated for either 5 min (closed symbols, continuous lines) or 10 min (open symbols, broken lines) at pH 7.0 in KCl-based media containing 2-oxoglutarate, L-malate, EGTA-Ca buffers and 1 mM-pyruvate as described for Fig. 1, together with the following other additions: none ($\blacksquare$, $\square$), 10 mM-NaCl plus 1 mM- MgCl_2 ($\bullet$, $\circ$), or 1 μM -Ruthenium Red ($\blacktriangle$). Where 10 min and 5 min values were very similar, the former have been omitted. The values shown and error bars represent means $\pm$ S.E.M. for observations made on at least three different preparations of mitochondria. $\star$ Indicates a value which is significantly different ($P \leq$ at least 0.05) from the $\star$ appropriate value indicated.

achieved with HEDTA-Ca instead of EGTA-Ca buffers (except that HEDTA-Ca buffers were not used with Mg^{2+} present, owing to the closeness between dissociation constants of HEDTA for Ca^{2+} and Mg^{2+} ; Martell & Smith, 1974). Effects of Ca^{2+} could also be mimicked by Sr^{2+} , though approx. 10-fold higher concentrations of Sr^{2+} were required in each case (results not shown). It should also be noted that total PDH activity in the mitochondria was not affected by the treatments used.

The results shown in Fig. 1 and Table 2 were obtained with mitochondria incubated for 5 min. Over the concentration ranges of Ca^{2+} used, essentially similar

Fig. 3. Effects of Ca^{2+} on the rate of decarboxylation of 2-oxoglutarate by intact rat liver mitochondria

Mitochondria prepared from the livers of female rats were incubated at 30 °C and pH 7.3 and approx. 0.5 mg of protein/ml in 100 μl of KCl-based medium containing 0.5 mM-L-malate and the concentrations (obtained by using EGTA- Ca buffers as in Fig. 1) of Ca^{2+} shown (see the Experimental section) in a small test tube within a sealed vial (the latter containing 2-phenethylamine) and in the presence of: no further additions ($\blacktriangle$, $\bullet$, $\blacktriangledown$, $\blacklozenge$), 10 mM-NaCl plus 1 mM- MgCl_2 ($\blacksquare$), or 1 μM -Ruthenium Red ($\triangle$, broken line). After 5 min, ($\blacktriangle$) 25 μM -, ($\bullet$, $\blacksquare$, $\triangle$) 100 μM -, ($\blacktriangledown$) 400 μM - or ($\blacklozenge$) 2 mM-2-oxoglutarate was added in a volume of 5 μl in each case, and containing suitable amounts of 2-oxo[1- ^{14}C]glutarate, followed 2.5 min later by 50 μl of 20% (v/v) HClO_4 . Full details of the method used are given by McCormack (1985a). Values shown are means obtained from observations made on at least three different preparations of mitochondria; S.E.M. values were all within 15% of the values shown. Similar results were obtained with mitochondria from male rat livers (not shown); this was also the case for the experiments shown in Figs. 4–7. It should also be noted that essentially similar results could be obtained if the 2-oxoglutarate solutions were added after preincubation for 0.5, 1, 2, 3 or 8, instead of 5, min (results not shown).

results were obtained after 10 min, indicating that steady-state amounts of PDH_a were present (not shown in full, Fig. 2). Therefore it is most likely that there were also steady-state relationships between extra- and intra-mitochondrial $[\text{Ca}^{2+}]$ under these conditions, i.e. there was 'cycling' of Ca^{2+} ions across the mitochondrial inner membrane, but no net change of $[\text{Ca}^{2+}]$ in either compartment (see Carafoli, 1979; Nicholls & Crompton, 1980; Denton & McCormack, 1980). It should also be noted that mitochondrial ATP content was not altered by Ca^{2+} over the concentration ranges used in Fig. 1 and Table 2 (see Fig. 2). However, if extramitochondrial $[\text{Ca}^{2+}]$ was raised above these ranges, then there was an

apparent time-dependent diminution of the effect of Ca^{2+} on PDH_a , which was accompanied by a time-dependent decrease in mitochondrial ATP content (Fig. 2). This has been noted previously with rat heart mitochondria (Denton *et al.*, 1980; Hansford, 1981), and presumably the diminutions occur when the egress pathway(s) become saturated by matrix $[\text{Ca}^{2+}]$, so that the uptake pathway, which has a much greater potential V_{max} capacity than the egress pathways, will then drive net Ca^{2+} accumulation by the mitochondria (see McCormack & Denton, 1985). In the presence of Na^+ and Mg^{2+} (Fig. 2), this only appears to occur when the extramitochondrial concentration of Ca^{2+} exceeds the expected physiological range for the cytoplasm of normal, intact, mammalian cells (see Rasmussen & Barrett, 1984). However, it should be pointed out that isolated mitochondria from several different mammalian tissues have previously been shown to be able to maintain their ATP contents, and also to have unimpaired respiratory control and proton electrochemical potentials, under comparatively similar conditions for Ca^{2+} accumulation, i.e. where they had been exposed to $> 1 \mu\text{M}$ - Ca^{2+} , so that the egress pathways were saturated [see Nicholls & Crompton (1980) for a review]. Indeed, this maintenance of integrity could even be demonstrated after the mitochondria had accumulated relatively large amounts of Ca, often in excess of 100 nmol/mg of protein (Nicholls & Crompton, 1980). The major difference between these studies and the present experiments lies in the amount of Ca^{2+} available for accumulation. Thus presumably the reason why the deteriorations occurred in the present experiments (and those previous quoted above) was because, once the conditions for accumulation were achieved (see above), there was then a vast reservoir of Ca available from the EGTA buffer system (at 5 mM strength) continuously to replenish the free Ca^{2+} concentration, thus creating conditions that could lead to massive and deleterious accumulation. This is in contrast with the more limited, and either unbuffered or weakly buffered, amounts of Ca^{2+} made available for accumulation in the studies reviewed by Nicholls & Crompton (1980), where, in fact, effective buffering of extramitochondrial $[\text{Ca}^{2+}]$ by the mitochondria could then be demonstrated. It is noteworthy in this context that the deleterious effects described in the present study, although they still occurred to some extent, were not so severe or so rapid in onset if the strength of the EGTA in the buffer was lowered to 0.25 mM (it should also be noted that this change in buffer strength did not appear to effect the Ca^{2+} -sensitivity curves for the enzyme at all).

Assay of the Ca^{2+} -sensitive properties of OGDH within intact rat liver mitochondria by monitoring $^{14}\text{CO}_2$ production from 2-oxo[1- ^{14}C]glutarate

The Ca^{2+} -sensitive properties of OGDH within intact mitochondria from rat heart (Denton *et al.*, 1980) and both brown (McCormack & Denton, 1980) and white (Marshall *et al.*, 1984) adipose tissue can be readily demonstrated by simply measuring O_2 uptake by the mitochondria when the restrictions imposed on this by respiratory-chain activity are overcome either by using uncoupled mitochondria or by adding an excess of ADP. This is because, in these mitochondria under these conditions, the activity of OGDH appears to be the rate-limiting step for the oxidation of added 2-oxoglutarate (see the references just cited), and marked

Table 3. Kinetic parameters for the effects of Ca^{2+} on the rate of decarboxylation of 2-oxoglutarate by intact rat liver mitochondria

Results were calculated and are expressed as for Table 1. Incubation conditions and the methods used were as described for Fig. 3, together with additions or changes as shown.

Basic incubation conditions	Other additions or changes	K_m for 2-oxoglutarate (μM) at:		$K_{0.5}$ value for Ca^{2+} (nM)
		$[\text{Ca}^{2+}] < 0.1 \text{ nM}$	$[\text{Ca}^{2+}] \approx 470 \text{ nM}$	
0.5 mM-L-Malate	None	1290 $\pm$ 102 (42)	98 $\pm$ 12 (38)	121 $\pm$ 29 (36)
	10 mM-NaCl	1040 $\pm$ 212 (28)	—	202 $\pm$ 36 (26)
	1 mM-MgCl ₂	980 $\pm$ 110 (26)	—	420 $\pm$ 58 (24)
	10 mM-NaCl + 1 mM-MgCl ₂	1140 $\pm$ 160 (28)	—	560 $\pm$ 48 (36)
No malate	None	1070 $\pm$ 148 (24)	142 $\pm$ 32 (22)	98 $\pm$ 14 (18)
	0.5 mM-Malonnate	222 $\pm$ 31 (20)	41 $\pm$ 7 (20)	86 $\pm$ 17 (18)
	0.5 mM-Malonnate + 0.5 mM-NH ₄ Cl	365 $\pm$ 48 (18)	62 $\pm$ 16 (18)	100 $\pm$ 14 (18)
	1 mM-Succinate	1540 $\pm$ 221 (16)	240 $\pm$ 48 (16)	116 $\pm$ 21 (16)
0.5 mM-L-Malate	At pH 7.1	590 $\pm$ 68 (18)	62 $\pm$ 11 (18)	105 $\pm$ 18 (18)

effects of Ca^{2+} on the oxidation of non-saturating concentrations of 2-oxoglutarate can be easily observed. However, when this direct approach was attempted with intact rat liver mitochondria, very poor results were obtained in terms of trying to demonstrate the effects of Ca^{2+} on OGDH within these mitochondria (results not shown). The K_m values for the oxidation of added 2-oxoglutarate (with or without L-malate or malonnate present) were found to be rather low (compared with these other types of mitochondria mentioned above), about 50–100 μM , and, at best, saturating concentrations of Ca^{2+} produced an approx. 1.5-fold stimulation of the rates of oxidation of non-saturating concentrations of 2-oxoglutarate. However, even this was not always observed and, in general, the rates of oxidation owing to added 2-oxoglutarate were much more difficult to study in the liver mitochondria (compared with the other types noted above), largely because of the much higher ratios of background rates to 2-oxoglutarate-dependent rates which were evident in the liver mitochondria (especially in the presence of malate). Attempts to assay the Ca^{2+} -sensitive properties of NAD-ICDH within the rat liver mitochondria in this way by monitoring O_2 uptake owing to added *threo*-D₃-isocitrate, as has been done successfully with both brown- and white-adipose-tissue mitochondria previously (McCormack & Denton, 1980; Marshall *et al.*, 1984), gave similar results to those for liver OGDH noted above (results not shown).

The Ca^{2+} -sensitive properties of OGDH within intact rat liver mitochondria could, however, be clearly demonstrated by incubating them in the absence of ADP or uncoupler, and measuring the production of $^{14}\text{CO}_2$ from 2-oxo[1- ^{14}C]glutamate at non-saturating concentrations of 2-oxoglutarate and in the presence of different concentrations of extramitochondrial Ca^{2+} (Fig. 3; see also McCormack, 1985a). Effects of Ca^{2+} could be observed under several different conditions (Fig. 3, Table 3) and were blocked by Ruthenium Red (Fig. 3). As expected, however, Ca^{2+} had no effects on the rate of $^{14}\text{CO}_2$ production at a saturating concentration of 2-oxoglutarate. The expected effects of Na^+ and Mg^{2+} on the $K_{0.5}$ values for Ca^{2+} were readily evident when this technique was used (Fig. 3, Table 3), and were similar to those noted in Fig. 1 and Table 2. It should be noted that $K_{0.5}$ values similar to those given (Table 3) could also be

obtained at different non-saturating concentrations of 2-oxoglutarate (see Fig. 3). The stimulatory effect of a modest decrease in pH on OGDH activity (at all concentrations of Ca^{2+}) was also demonstrated (Table 3), and again Sr^{2+} could be shown to mimic Ca^{2+} action as before (results not shown).

No effects of Ca^{2+} were observed, by using this method, in the presence of uncoupler or excess ADP when assayed at several different concentrations of 2-oxoglutarate. This suggests, as above, that some feature other than OGDH becomes rate-limiting for the oxidative of 2-oxoglutarate by rat liver mitochondria when the respiratory chain is fully stimulated.

The stimulation by Ca^{2+} of the rate of $^{14}\text{CO}_2$ production from [1- ^{14}C]glutamate could also be observed by this technique, although the K_m values for glutamate were approx. 5 times those observed for 2-oxoglutarate (results not shown). This is presumably because of the effects of Ca^{2+} on OGDH. Unfortunately NAD-ICDH could not be studied satisfactorily with a similar technique owing to problems associated with the mitochondria having a high activity of NADP⁺-linked isocitrate dehydrogenase, and isotope exchange.

The raising of extramitochondrial $[\text{Ca}^{2+}]$ above the ranges depicted in Fig. 3 (i.e. as in Fig. 2) caused diminutions in the rate of $^{14}\text{CO}_2$ production (results not shown); however, this was evident at both non-saturating and saturating [2-oxoglutarate].

Assay of the Ca^{2+} -sensitive properties of OGDH and NAD-ICDH within intact rat liver mitochondria by monitoring NAD(P)H production with a dual-wavelength spectrophotometer

The Ca^{2+} -sensitive properties of OGDH within intact rat heart mitochondria can also be demonstrated by measuring the reduction of NAD(P)⁺ by added 2-oxoglutarate, by using either a fluorimeter (Hansford & Castro, 1981) or a dual-wavelength spectrophotometer (McCormack *et al.*, 1982), each of which was fitted with thermostatically controlled cuvette units in which the samples could be continuously stirred. These previous experiments were performed either in the absence of ADP or uncoupler, or else in their initial presence (to enhance the rate of oxidation of endogenous substrates), before 2-oxoglutarate-induced NAD(P)⁺ reduction was meas-

Fig. 4. Effects of Ca^{2+} on the extent of NAD(P)^+ reduction induced by 2-oxoglutarate in intact rat liver mitochondria

Mitochondria prepared from female rat livers were incubated (with stirring in a cuvette) at 30 °C and pH 7.3 and approx. 1.5 mg of protein/ml, and for the times shown, in 2 ml of KCl-based medium (see the Experimental section) containing initially 0.5 mM-EGTA and: A, no further additions; B, 0.5 mM-malonnate; or C, 0.5 mM-malonnate plus 0.5 mM- NH_4Cl . In each case, at arrow a either (i) 2 mM-EGTA or (ii) 2 mM-EGTA plus 2 mM- CaCl_2 (resultant free $[\text{Ca}^{2+}]$ approx. 200 nM) was added, except for the broken traces, where no addition was made at arrow a. In (a) other additions were, at the arrows, as follows: b, 50 μM -2-oxoglutarate; c, 2 mM-2-oxoglutarate; d, 0.5 μg of rotenone. In (b) other additions were: b, 200 μM -2-oxoglutarate; c, either (continuous lines) 2 mM-2-oxoglutarate, or (broken lines): 1, 5 mM-EGTA plus 5 mM- CaCl_2 (resultant free $[\text{Ca}^{2+}]$ approx. 470 nM); 2, 3.5 mM-EGTA plus 3.5 mM- CaCl_2 ($\approx$ 340 nM); 3, 1.5 mM-EGTA plus 1.5 mM- CaCl_2 ($\approx$ 150 nM); 4, 1 mM-EGTA plus 1 mM- CaCl_2 ($\approx$ 90 nM); 5, 0.5 mM-EGTA plus 0.5 mM- CaCl_2 ($\approx$ 50 nM); or 6, 0.5 mM-EGTA alone; d, 0.5 μg of rotenone; e, 2 mM-ADP. Results from a typical experiment are shown (but see also Fig. 5 and Table 4). Note that 2-oxoglutarate itself absorbed at the wavelength pair used; this has been omitted for clarity of presentation, and only NAD(P)^+ reduction is shown.

ured in the additional presence of respiratory-chain inhibitors (e.g. antimycin A), or when the ADP was used up, so that conditions were favourable for NAD(P)H build-up (see references just cited).

Fig. 4(a) (curve A) shows that with intact rat liver mitochondria only a small amount of oxidation of the endogenous substrate appeared to occur in the absence of ADP or uncoupler; thus the extent of reduction owing to added 2-oxoglutarate was comparatively small. However, effects of Ca^{2+} could be observed under these conditions, although the K_m values for 2-oxoglutarate were much lower than those for the heart (Hansford & Castro, 1981; McCormack *et al.*, 1982). There was also a large degree of variability in the extent of initial oxidation which took place, both from day to day and within any one day, making quantification of results difficult. The initial addition of ADP or uncoupler did result in much more oxidation [about 4-fold that of Fig. 4(a), curve A; results not shown]; however, when respiratory-chain inhibitors were then added, or the ADP was used up, a large and variable degree of reduction then occurred and again the apparent K_m values for 2-oxoglutarate were very low, making effects of Ca^{2+} difficult to observe. Yet very little 2-oxoglutarate-induced reduction at all took place in the presence of uncoupler or ADP. The addition of malonnate [Fig. 4(a), curve B] improved the situation; however, by far the most convenient system found was to add malonnate together

with a small amount of NH_4Cl (Fig. 4b) to cause oxidation of endogenous NAD(P)H . Under these conditions much larger degrees of reduction could be induced by adding 2-oxoglutarate, and the apparent K_m values for 2-oxoglutarate were much higher. This meant that the resultant extents of NAD(P)^+ reduction were much more reproducible and stable, as it appeared that steady-state degrees of reduction could be attained (Fig. 4b). The NAD(P)H formed owing to the added 2-oxoglutarate could be re-oxidized by adding ADP (Fig. 4b). The addition of rotenone caused a small amount of further reduction in the presence of saturating concentrations of 2-oxoglutarate (Fig. 4). Changes in the concentration of NH_4Cl caused changes in the rate of initial oxidation and in the apparent K_m values for 2-oxoglutarate (results not shown); 0.5 mM- NH_4Cl was found to be a convenient concentration to use [Fig. 4(b), Table 4]. The addition of Ca^{2+} before the mitochondria sometimes [especially under the conditions used for Fig. 4(a)] caused a lowering in the rate of initial oxidation (not shown); this would be expected if, e.g., some 2-oxoglutarate was among the endogenous substrates. However, the addition of Ca^{2+} from about 2–3 min onwards after the mitochondria (as in Fig. 4) did not alter the rate of oxidation, or cause any reduction, in the absence of 2-oxoglutarate.

Clear effects of Ca^{2+} on both the initial rate and final extent of NAD(P)^+ reduction owing to the addition of

Table 4. Kinetic parameters for the effects of Ca^{2+} on the reduction of NAD(P)^+ induced by several different substrates in intact rat liver mitochondria

Results were calculated and are expressed as for Table 1. Incubation conditions and the methods used were as described for Fig. 4 (together with the additions or changes shown below), except that other substrates, as well as 2-oxoglutarate, were used to induce NAD(P)^+ reduction as indicated. In general, parameters were calculated from experimental values where the extents of NAD(P)^+ reduction achieved under the desired conditions of [substrate] and $[\text{Ca}^{2+}]$ were expressed as the percentages of the maximal extents of NAD(P)^+ reduction that could be achieved with the appropriate substrate (i.e. at saturating [substrate]): * in one instance, the initial rates of NAD(P)^+ reduction were measured instead, but were expressed in a similar manner. Abbreviation: NA, not applicable.

Basic conditions	Substrate	Other additions or changes	K_m value for the appropriate substrate (μM) at:			$K_{0.5}$ value for Ca^{2+} (nM)
			$[\text{Ca}^{2+}] < 0.1 \text{ nM}$	$[\text{Ca}^{2+}] \approx 470 \text{ nM}$	$[\text{Ca}^{2+}] \approx 910 \text{ nM}$	
0.5 mM-Malonate + 0.5 mM- NH_4Cl	2-Oxoglutarate	None	1020 ± 120 (26)	190 ± 40 (26)	—	121 ± 14 (22)
		*None; rate measured	—	—	—	96 ± 8 (24)
		10 mM-NaCl	—	—	—	210 ± 19 (18)
		1 mM- MgCl_2	—	—	—	490 ± 38 (15)
		10 mM-NaCl + 1 mM- MgCl_2	1170 ± 204 (18)	—	265 ± 39 (15)	595 ± 63 (18)
Malonate alone	2-Oxoglutarate	None	360 ± 42 (18)	42 ± 8 (15)	—	131 ± 27 (15)
No malonate or NH_4Cl	2-Oxoglutarate	None	420 ± 36 (18)	74 ± 12 (18)	—	106 ± 11 (18)
0.5 mM-Malonate + 1 mM-MgATP	2-Oxoglutarate	None	1590 ± 220 (24)	—	360 ± 50 (24)	400 ± 76 (20)
		10 mM-NaCl	1410 ± 350 (15)	—	350 ± 32 (15)	560 ± 71 (18)
0.5 mM-Malonate + 0.5 mM- NH_4Cl	Glutamate	None	2620 ± 610 (15)	470 ± 72 (15)	—	126 ± 32 (15)
	β -Hydroxybutyrate	None	5280 ± 1080 (12)	4920 ± 840 (12)	—	NA
	L-Malate	None	265 ± 36 (12)	294 ± 52 (12)	—	NA
	<i>threo</i> - D_8 -Isocitrate	0.5 mM-Hydroxymalonate	136 ± 32 (15)	29 ± 6 (15)	—	98 ± 11 (16)
	Citrate	0.5 mM-Hydroxymalonate	1690 ± 260 (24)	202 ± 36 (24)	—	109 ± 9 (26)
		0.5 mM-Hydroxymalonate + 10 mM-NaCl + 1 mM- MgCl_2	1430 ± 160 (12)	—	190 ± 22 (12)	610 ± 95 (15)

non-saturating concentrations of 2-oxoglutarate were observed (Figs. 4 and 5); these were evident when 2-oxoglutarate was added either before or after Ca^{2+} (Fig. 4). The assessment of the Ca^{2+} effects by measuring either the initial rate or the final extent of NAD(P)^+ reduction gave essentially similar results (Fig. 5). Fig. 5 also shows that if extramitochondrial $[\text{Ca}^{2+}]$ was raised beyond the ranges wherein OGDH was activated, then once again there were apparent time-dependent deteriorations in enzyme activity at both non-saturating and saturating concentrations of 2-oxoglutarate. Fig. 6 shows that a time-dependent decrease in mitochondrial ATP content accompanied these decreases in NAD(P)H . This deterioration was also observed in the additional presence of rotenone and also with other substrates (results not known), including those whose induced reduction of NAD(P)^+ was not stimulated by Ca^{2+} (see Table 4).

The results presented earlier in Table 3 indicate that, under the same conditions where Ca^{2+} caused increases in the extent of 2-oxoglutarate-induced NAD(P)^+ reduction (as, e.g., in Fig. 6), there were also increases in the rate of 2-oxoglutarate decarboxylation. Fig. 6 shows that after the initial step for the NAD(P)^+ -reduction experiments the mitochondrial ATP content was very low (with or without Ca^{2+}) and that, subsequently, the Ca^{2+} -stimulated 2-oxoglutarate decarboxylation and NAD(P)^+ reduction were also accompanied by Ca^{2+} -stimulated ATP production by the mitochondria. Therefore, it is very unlikely that the observed effects of

Ca^{2+} were brought about by its causing changes in the concentrations of any of the other effectors of the enzyme (see McCormack & Denton, 1979). In addition, the stimulatory effects of Ca^{2+} on NAD(P)^+ reduction could all be blocked by Ruthenium Red (results not shown).

The expected effects of Na^+ and Mg^{2+} on the $K_{0.5}$ values for Ca^{2+} could also be demonstrated by this technique (not shown in full, Table 4). Table 4 also shows that the extent of NAD(P)^+ reduction induced by adding non-saturating concentrations of glutamate could also be stimulated by Ca^{2+} , although the K_m values for glutamate were higher than those for 2-oxoglutarate. In contrast, the reductions of NAD(P)^+ induced by either non-saturating or saturating concentrations of β -hydroxybutyrate or of L-malate were unaffected by Ca^{2+} (Table 4), even though effects of Ca^{2+} on subsequently added 2-oxoglutarate could be readily observed, provided that NAD(P)^+ reduction was not saturated by the prior presence of either of the former substrates (results not shown). Clear effects of Ca^{2+} could also be observed on the reduction of NAD(P)^+ induced by non-saturating concentrations of *threo*- D_8 -isocitrate (Table 4). The apparent K_m values for *threo*- D_8 -isocitrate under the conditions used were quite low (Table 4), and it was therefore found to be much easier to use citrate as a substrate in investigating Ca^{2+} effects on NAD-ICDH with this technique, as the final extents of NAD(P)^+ reduction were much more stable, and more measurable initial rates of reduction were achieved, with the latter

Fig. 5. Effects of increasing extramitochondrial $[\text{Ca}^{2+}]$ on (a) the extent, and (b) the rate and extent, of NAD(P)⁺ reduction induced by either (a) a saturating, or (b) a non-saturating, concentration of 2-oxoglutarate in rat liver mitochondria

Mitochondria were incubated initially as described for Fig. 4(b), but at pH 7.0 and in the presence of only 0.05 mM-EGTA. Suitable (buffered) concentrations of EGTA and CaCl_2 were added after 4.5 min to give the concentrations of Ca^{2+} shown. Then 30 s later either ($\blacktriangle$, $\blacktriangledown$, $\triangle$) 250 μM - or ($\bullet$, $\circ$) 4 mM-2-oxoglutarate was added and either the resultant initial rate ($\blacktriangledown$) or the extent ($\blacktriangle$, $\triangle$, $\bullet$, $\circ$) of NAD(P)⁺ reduction was measured. The latter values were measured at either the maximal extent attained at the indicated $[\text{Ca}^{2+}]$ ($\blacktriangle$, $\bullet$) or at 2.5 min after this maximal value was first attained ($\triangle$, $\circ$) (see Fig. 6 below); where these two different time values were very similar [i.e. at an apparent steady-state extent of NAD(P)⁺ reduction], the latter has been omitted for clarity of presentation. Values shown are means of observations made on at least three different preparations of mitochondria; S.E.M. values were all $\leq 15\%$ of the values shown.

(not shown in full, Table 4). In addition, by using ADP to reoxidize NAD(P)H (as in Fig. 4), some evidence was obtained that when *threo*-D₅-isocitrate was used a considerable amount of NADPH was being formed, presumably by the NADP⁺-linked isocitrate dehydrogenase (results not shown); this did not appear to be such a problem when citrate was used. However, it should be noted that the amount of ADP-oxidizable NAD(P)H formed at a suitable [*threo*-D₅-isocitrate] was sufficient probably to discount the possibility that the effects of Ca^{2+} observed were simply due to the subsequent activity of OGDH.

During initial experiments endeavouring to ascertain the best conditions for examining NAD(P)H formation in liver mitochondria, it was discovered that the addition of some MgATP led to an oxidation of endogenous NAD(P)H to a degree similar to that was achieved by the addition of ADP (Fig. 7a). However, in this case, and in contrast with the latter situation [i.e. with ADP; not

Fig. 6. Effects of Ca^{2+} on (a) the ATP content of, and (b) the reduction of NAD(P)⁺ in, rat liver mitochondria incubated with non-saturating and saturating concentrations of 2-oxoglutarate

Mitochondria were incubated initially as described for Fig. 4(b), but with only 0.1 mM-EGTA; the 0 min time point in the present Figure is equivalent to the 5 min time point in Fig. 4(b). For both (a) and (b), at arrow (i) 2.4 mM-EGTA ($\blacktriangledown$) or (ii) 0.5 mM-EGTA plus 0.5 mM- CaCl_2 ($\blacktriangle$; resultant free $[\text{Ca}^{2+}] \approx 240$ nM) were added, followed by: b, 50 μM -2-oxoglutarate; c, 300 μM -2-oxoglutarate; d, 4 mM-2-oxoglutarate. For the upper [i.e. (ii)] traces only, and for (a) ($\triangle$) and (b) (broken trace), a further 2.5 mM-EGTA plus 2.5 mM- CaCl_2 (resultant free $[\text{Ca}^{2+}] \approx 1020$ nM) was added at arrow e. A typical experiment is shown in (b). The values in (a) are means of observations made on samples, taken at the indicated times, from three different preparations of mitochondria subjected to the experiment shown in (b), including the one illustrated.

shown in full, Fig. 7(b)], the rate and extent of NAD(P)⁺ reduction induced by non-saturating concentrations of 2-oxoglutarate (added after the MgATP) could be stimulated by Ca^{2+} (Fig. 7). This Ca^{2+} -sensitivity was observed after an apparent Ca^{2+} -insensitive initial phase of NAD(P)⁺ reduction (Figs. 7a and 7c). The duration of this Ca^{2+} -insensitive phase, together with the initial rates of oxidation, the apparent K_m values for 2-oxoglutarate, and the final extents of NAD(P)⁺ reduction, were all dependent on the concentration of MgATP used (results

Fig. 7. Effects of Ca^{2+} on the reduction of NAD(P)^+ induced by 2-oxoglutarate in intact rat liver mitochondria incubated under 'State 3.5' conditions achieved in the presence of MgATP

Mitochondria were incubated as in Fig. 4 but in the initial presence of 0.5 mM-malonate, 0.1 mM-EGTA and 1 mM-MgATP. In (a), at arrow a either (i) 4.9 mM-EGTA or (ii) 4.9 mM-EGTA plus 4.5 mM- CaCl_2 (resultant free $[\text{Ca}^{2+}] \approx 520 \text{ nM}$) was added, except for the broken traces, where no addition was made at arrow a. For (a), other additions were: b, 400 μM -2-oxoglutarate; c, either (continuous lines) 2 mM-2-oxoglutarate or (broken lines) 4.9 mM-EGTA plus: 1, 4.9 mM- CaCl_2 ($[\text{Ca}^{2+}] \approx 2750 \text{ nM}$); 2, 4.7 mM- CaCl_2 (910 nM); 3, 4.5 mM- CaCl_2 (520 nM); 4, 4.2 mM- CaCl_2 (320 nM); 5, 3.5 mM- CaCl_2 (130 nM); or 6, no CaCl_2 ; d, 0.5 μg of rotenone; e, 2 mM-ADP; f, 1 mM- NH_4Cl . In (b) 4.9 mM-EGTA plus 4.2 mM- CaCl_2 (320 nM) was added 0.5 min before the start of the trace shown, and at arrow a 300 μM -2-oxoglutarate was added either with (i) or without (ii) 2 mM-ADP; in (ii) the broken lines indicate the effects of adding 2 mM-ADP at the times indicated by arrows b, except that arrow c indicates the addition of 1 mM- NH_4Cl . In (c) 4.9 mM-EGTA either (i) alone or (ii) with 4.5 mM- CaCl_2 (520 nM) were added 0.5 min before the start of the traces shown, and 350 μM -2-oxoglutarate was added at arrow a; at arrow b, either (continuous lines) 1 mM- NH_4Cl or (broken lines) 2 mM-arsenite was added, with 2 mM-ADP being added at c. Typical traces are shown (but see Table 4).

not shown); a concentration of 1 mM was found to be convenient (Fig. 7, Table 4). This initial phase of Ca^{2+} -insensitive reduction appears to be due to the presence of excess (i.e. saturating) ADP during the initial period after 2-oxoglutarate addition, as it could be extended by adding ADP (Fig. 7b). This conclusion was supported by observations, made under the conditions used in Fig. 7, with an oxygen electrode. Thus the addition of 2-oxoglutarate in the presence of ATPMg initially resulted in a rate of O_2 uptake identical with that in the presence of ADP (i.e. State 3 respiration; Chance & Williams, 1956). However, in the former case the rate then, after 1 or 2 min and coincident with the development of the Ca^{2+} -sensitivity as shown in Fig. 7, gradually declined to a slightly slower rate (i.e. 'State 3.5' respiration), which was constant from about 3–4 min onwards. In the absence of ADP or MgATP the rate of O_2 uptake was the same in the presence or absence of 2-oxoglutarate and was $7.5 \pm 0.9 \text{ nmol of O}_2/\text{min per mg}$ of protein (mean $\pm$ S.E.M. for three different preparations of mitochondria). In the presence of ADP, the corresponding rates at 350 μM - and 5 mM-2-oxoglutarate were 23.2 ± 4.2 and 28.7 ± 3.1 respectively, which were similar to the initial rates achieved in the presence of ATPMg. However, these latter (see above) then declined to new linear rates (measured from 4 min after 2-oxoglutarate addition) of 17.8 ± 3.5 and

$23.8 \pm 4.6 \text{ nmol/min per mg}$ respectively; in the presence of ADP the rate of O_2 uptake with 20 mM-ascorbate plus 0.5 mM- $\text{NNN}'\text{N}'$ -tetramethylphenylenediamine was 303 ± 29 under these conditions. It should be noted that no effects of Ca^{2+} on these rates of O_2 uptake were evident.

During this latter phase of 'State 3.5' respiration, it would appear that NAD(P)H can build up sufficiently to allow the effects of Ca^{2+} on OGDH to be adequately demonstrated by this technique (Fig. 7). This phase presumably arises because in the preparation there is some MgATPase which has activity sufficient to allow small, non-saturating, concentrations of ADP to be maintained under these conditions. If, in experiments of the type described in Fig. 3 but performed in the presence of ATPMg as in Fig. 7, some radioactive 2-oxoglutarate was added at a point equivalent to arrow b in Fig. 7(a), then no effects of Ca^{2+} on $^{14}\text{CO}_2$ production (collected over the next 2.5 min) were observed (results not shown); in contrast, if a trace of radioactive 2-oxoglutarate was added at arrow c (i.e. about 4 min after 400 μM unlabelled 2-oxoglutarate was added), then effects of Ca^{2+} were readily evident.

Fig. 7(a) shows that most of the NAD(P)H formed owing to 2-oxoglutarate addition could be re-oxidized with ADP and that the amount of ADP-induced re-oxidation was larger when larger amounts of

NAD(P)H were formed owing to the presence of Ca^{2+} . After ADP (Fig. 7a), the remainder could be re-oxidized by NH_4Cl , but Ca^{2+} had no effect on the amount of re-oxidation in this instance. Fig. 7(c) shows that, if NH_4Cl was added before ADP, the extent of re-oxidation that could be achieved was similar in the presence or absence of Ca^{2+} . However, the initial rate of re-oxidation was much slower in the presence of Ca^{2+} in this case; this is presumably because more NADH was being made, owing to the enhanced activity of OGDH under these circumstances. An alternative possibility is that Ca^{2+} was making the NAD(P)H more stable within the mitochondria. However, in addition to the results obtained with 2-oxo ^{14}C glutarate noted in the preceding paragraph, Fig. 7(c) shows that if arsenite (a potent inhibitor of OGDH; Sanadi *et al.*, 1959) was added, then (after a small lag) the rate of re-oxidation of NAD(P)H was faster in the presence of Ca^{2+} , presumably because more NAD(P)H was present initially as the result of Ca^{2+} effects on OGDH.

General discussion and conclusions

The major conclusion of the present paper is that the three Ca^{2+} -sensitive intramitochondrial dehydrogenases within intact rat liver mitochondria incubated with physiological concentrations of Na^+ and Mg^{2+} [see, e.g., Lee & Fozzard (1975) and Hutson (1977) respectively] can be activated by increasing the extramitochondrial concentration of Ca^{2+} within the expected physiological range, i.e. approx. $0.05\text{--}5\text{ }\mu\text{M}$ (see, e.g., Rasmussen & Barrett, 1984). This therefore lends further credence to the proposal (based on observations made with other types of mammalian mitochondria) that Ca^{2+} ions are likely to be important regulators of intramitochondrial metabolism through their effects on these key enzymes. The present work is also important in arguing against the contradictory proposals (see, e.g., Williamson *et al.*, 1981; Joseph *et al.*, 1983) that the concentration of Ca^{2+} within liver mitochondria would always be above the range to which the enzymes show Ca^{2+} -sensitivity. However, it is appreciated that the demonstration of the Ca^{2+} -sensitivity of these enzymes within liver mitochondria was not so straightforward or simple as it had been within these other types of mitochondria; the reasons for this have been discussed in the preceding section.

There have been reports that Ca^{2+} may also inhibit carbamoyl phosphate synthase (Cerdan *et al.*, 1984) and pyruvate carboxylase (Wimhurst & Manchester, 1970) for liver mitochondria. However, the physiological significance of these findings remains to be established, especially as Ca^{2+} only affects the enzymes at rather high $[\text{Ca}^{2+}]$ (at least an order of magnitude higher than the range affecting the dehydrogenases) and also because the Ca^{2+} effects on these two enzymes appear to be greatly diminished in the presence of physiological concentrations of Mg^{2+} .

The present work also substantiates the proposal by Denton & McCormack (1980, 1981) that, under normal physiological conditions in mammalian cells, the Ca^{2+} -transport systems of the mitochondrial inner membrane exist primarily to relay changes in cytoplasmic $[\text{Ca}^{2+}]$ to the mitochondrial matrix, rather than, conversely, to buffer or set cytoplasmic $[\text{Ca}^{2+}]$ (see, e.g., Puskin *et al.*, 1976; Nicholls, 1978; Becker *et al.*, 1980; Borle, 1981; Nedergaard, 1984). Further support for the former

proposal is provided in the following paper (McCormack, 1985b), which, by using the techniques developed in the present paper, and the strategy developed by McCormack & Denton (1984) for studying hormonal effects on intramitochondrial $[\text{Ca}^{2+}]$ in the heart, shows that hormones which are known to cause increases in liver cytoplasmic $[\text{Ca}^{2+}]$ also activate PDH and OGDH by apparently causing increases in intramitochondrial $[\text{Ca}^{2+}]$.

From studies in other laboratories, it would appear that the ranges of mitochondrial total Ca content over which the Ca^{2+} -sensitive matrix dehydrogenases can be regulated by intramitochondrial Ca^{2+} [approx. $0.5\text{--}3\text{ nmol}$ of Ca/mg of protein (Hansford & Castro, 1981; Coll *et al.*, 1982)] and those at which the mitochondria can buffer or set extramitochondrial $[\text{Ca}^{2+}]$ [approx. $\geq 10\text{ nmol/mg}$ of protein (see, e.g., Nicholls & Åkerman, 1982)] are mutually exclusive. Significantly, it is the former range which agrees better with measurements *in situ* of total mitochondrial Ca content of 'snap-frozen' pieces of various tissues by using X-ray probe micro-analysis (see Somlyo *et al.*, 1981), and also with the most recent estimates of the content of Ca in rat liver mitochondria *in vivo* by using indirect techniques [approx. $0.5\text{--}2\text{ nmol/mg}$ of protein (Reinhart *et al.*, 1984; Kleineke & Söling, 1985)]. In a very recent article, Somlyo *et al.* (1985) report a value of 1.1 nmol of Ca/mg of protein for liver mitochondria *in situ*, using the X-ray probe micro-analysis technique. In addition it is worth noting that most workers have found that the set-point at which isolated mitochondria will buffer extramitochondrial $[\text{Ca}^{2+}]$ is around $1\text{ }\mu\text{M}$ in the presence of either Mg^{2+} or Na^+ (see, e.g., Brand & DeSelincourt, 1980; Hughes *et al.*, 1980; Nicholls & Åkerman, 1982; Fiskum & Lehninger, 1982; Nedergaard, 1984) and, as would be expected, is even higher if both Mg^{2+} and Na^+ are present (see, e.g., Fry *et al.*, 1984). However, it should be pointed out that some authors have found set-point values as low as $0.3\text{--}0.4\text{ }\mu\text{M}$ in the presence of either Na^+ or Mg^{2+} alone [see the review by Fiskum & Lehninger (1982) for examples], which, of course, is within the expected physiological range for extramitochondrial $[\text{Ca}^{2+}]$. Nevertheless, in general it would appear that in the presence of both Na^+ and Mg^{2+} mitochondria may only be able to set extramitochondrial $[\text{Ca}^{2+}]$ at a concentration which is at the highest end of, or above, the expected physiological range. This suggests that in normal cells other Ca^{2+} -binding entities (e.g. reticulum membranes) will set cytoplasmic $[\text{Ca}^{2+}]$. Strong evidence to support this view is provided by Burgess *et al.* (1983), who showed that, in permeabilized hepatocytes incubated with MgATP and physiological concentrations of extramitochondrial Ca^{2+} , it is apparently a reticulum fraction which is the predominant Ca pool and that it is only if extramitochondrial $[\text{Ca}^{2+}]$ exceeds $1\text{ }\mu\text{M}$ that accumulation of Ca^{2+} by mitochondria occurs. Thus it may be that the ability of mitochondria to accumulate Ca^{2+} and hence buffer extramitochondrial $[\text{Ca}^{2+}]$ (at or slightly above about $1\text{ }\mu\text{M}$) exists primarily as a defensive mechanism to prevent cell damage and is reserved for situations after abnormal Ca^{2+} flux across the plasma membrane, as it is only under such circumstances that cytoplasmic Ca^{2+} concentrations in excess of the set-point value are likely to arise. However, it has been pointed out earlier in the Results and discussion section that this function may not be limitless and that the excessive accumulation of Ca^{2+}

in this way may lead to diminution of mitochondrial function, even though it has been established that mitochondria appear to be able to retain their bioenergetic functions with Ca loads in excess of 100 nmol/mg of protein (see Nicholls & Crompton, 1980). Notwithstanding the latter observations, it is suggested that the inhibition of the oxidation of an apparently saturating (2 mM) concentration of 2-oxoglutarate by rat liver mitochondria reported by Borrebaek *et al.* (1984) may have been the result of using too high concentrations of extramitochondrial Ca^{2+} ; in the presence of Mg^{2+} (but no Na^+) inhibition did not occur until extramitochondrial $[\text{Ca}^{2+}]$ exceeded $1\ \mu\text{M}$. These authors, and also Ontko & Westbrook (1983), have also observed an inhibition of palmitate oxidation under similar conditions.

Another major reason for arguing against a role for the Ca^{2+} -transport systems of the mitochondrial inner membrane in setting cytoplasmic $[\text{Ca}^{2+}]$ (under normal physiological conditions) is that, for this behaviour to occur, the egress pathways for Ca^{2+} from mitochondria are required to be saturated for internal Ca^{2+} (see Nicholls & Crompton, 1980; Nicholls & Åkerman, 1982), otherwise changes in matrix $[\text{Ca}^{2+}]$ and extramitochondrial $[\text{Ca}^{2+}]$ will occur in parallel [see McCormack & Denton (1985) and also Joseph *et al.* (1983)]. In the presence of Na^+ , the egress pathways only appear to be saturated at or above about 10 nmol of Ca/mg of protein (see Nicholls & Crompton, 1980; Nicholls & Åkerman, 1982). The observed increases in enzyme activities as the result of increasing extramitochondrial $[\text{Ca}^{2+}]$ in the present paper are most likely to result from broadly parallel increases in matrix $[\text{Ca}^{2+}]$ under conditions where the egress pathways are not saturated.

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