Studies on the activation of rat liver pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase by adrenaline and glucagon

Role of increases in intramitochondrial Ca^{2+} concentration

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1. The administration *in vivo* of either adrenaline or glucagon alone resulted in increases of about 2-fold in the amounts of active, non-phosphorylated, pyruvate dehydrogenase in the livers of fed male or female rats, whereas when administered together increases of about 4-fold were obtained. 2. Ca^{2+} -dependent increases in the amount of active enzyme of up to about 5-fold could be achieved in isolated rat liver mitochondria by incubating them with increasing extramitochondrial $[\text{Ca}^{2+}]$; from this, two conditions of Ca loading were chosen which caused increases in active enzyme similar to those with the hormone treatments given above. 3. The increases in enzyme activity owing to these Ca loads persisted through the 're-isolation' of mitochondria and their incubation in Na^+ -free KCl-based media containing EGTA. Differences from values obtained with unloaded controls could be diminished by adding Na^+ ions to cause the egress of Ca^{2+} from the mitochondria, or enough extramitochondrial Ca^{2+} to saturate the enzyme in its Ca^{2+} -dependent activation; the effects of Na^+ could be blocked by diltiazem, an inhibitor of mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchange. 4. The re-isolated, Ca-preloaded, mitochondria also exhibited enhanced activities of 2-oxoglutarate dehydrogenase when assayed at non-saturating [2-oxoglutarate] by two different methods; effects of Na^+ , Ca^{2+} or diltiazem on the persistent activations of this enzyme were similar to those for pyruvate dehydrogenase. 5. Na^+ caused a marked depletion, which could be blocked by diltiazem, of the ^{45}Ca content of re-isolated mitochondria which had pre-loaded with Ca, containing ^{45}Ca , to the same degrees as above. 6. The activities of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase in incubated liver mitochondria prepared from rats subjected to the hormone treatments given above were found to behave in a very similar manner to those exhibited in the re-isolated, Ca-preloaded, mitochondria. 7. It is concluded that these hormones each bring about the activations of these rat liver enzymes by causing increases in intramitochondrial $[\text{Ca}^{2+}]$, and that their effects, as such, are additive.

INTRODUCTION

The preceding paper (McCormack, 1985b) describes means whereby the effects of Ca^{2+} on the intramitochondrial Ca^{2+} -sensitive dehydrogenases (pyruvate dehydrogenase, NAD^+ -isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase; see Denton & McCormack, 1980) can be demonstrated within intact rat liver mitochondria. Moreover it shows that, in intact, fully coupled, liver mitochondria incubated with physiological concentrations of Na^+ and Mg^{2+} , these enzymes can all be activated by increases in the extramitochondrial concentration of Ca^{2+} within the expected physiological range (i.e. approx. $0.05\text{--}5\ \mu\text{M}$).

Similar observations have been made previously on intact mitochondria isolated from rat heart (Denton *et al.*, 1980; Hansford, 1981; Hansford & Castro, 1981), skeletal muscle (Ashour & Hansford, 1983; Fuller & Randle, 1984), epididymal adipose tissue (Marshall *et al.*, 1984) and brain (Hansford & Castro, 1985), and within permeabilized pig lymphocytes (Baumgarten *et al.*, 1983). Such observations led Denton & McCormack (1980, 1981) to propose that hormones and other extracellular

agents which are known to affect cytoplasmic Ca^{2+} -dependent processes by causing increases in cytoplasmic $[\text{Ca}^{2+}]$ (within this concentration range) may thus bring about increases in intramitochondrial $[\text{Ca}^{2+}]$ and so activate these key enzymes of mitochondrial oxidative metabolism.

McCormack & Denton (1984) have obtained strong evidence in support of this hypothesis in studies on the effects on the rat heart of positive inotropic agents (which cause increases in cytoplasmic $[\text{Ca}^{2+}]$). In particular it was found that mitochondria prepared from hearts treated with adrenaline exhibited enhanced activities of both enzymes when incubated at $30\ ^\circ\text{C}$, in the absence of Na^+ , in KCl-based media containing EGTA. Moreover, these differences from mitochondria prepared from untreated hearts could be abolished by adding either Na^+ ions to stimulate Ca^{2+} egress from the mitochondria (see Carafoli, 1979; Nicholls & Crompton, 1980) or enough extramitochondrial Ca^{2+} to lead to the saturation of the Ca^{2+} -dependent activations of the enzymes. In addition, these effects of Na^+ were blocked by diltiazem {*cis*-(+)-3-acetyloxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-

Abbreviation used: PDH_a, the active, non-phosphorylated, form of the pyruvate dehydrogenase complex.

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one), a potent inhibitor of Na^+ -induced egress of Ca^{2+} from heart mitochondria (Vághy *et al.*, 1982).

Several hormones are known to cause increases in $[\text{Ca}^{2+}]$ in the cytoplasm of liver cells, which have now been demonstrated directly by using Quin 2, including adrenaline, vasopressin, angiotensin and glucagon (see, e.g., Charest *et al.*, 1983; Berthon *et al.*, 1984; Thomas *et al.*, 1984). These hormones are also known to bring about increases in the amount of the active, non-phosphorylated, form of the pyruvate dehydrogenase complex (PDH_a) in perfused livers or isolated hepatocytes from fed rats (Hems *et al.*, 1978; Assimacopoulos-Jeannet *et al.*, 1983; Sies *et al.*, 1983; Oviyasu & Whitton, 1984). In addition, Assimacopoulos-Jeannet *et al.* (1983) have reported that the effects of glucagon on PDH_a appeared to be additive with those of the α -adrenergic agonist phenylephrine when each hormone was added at a maximum effective concentration. There is some indirect evidence that these activations of pyruvate dehydrogenase may involve increases in intramitochondrial $[\text{Ca}^{2+}]$. Thus incubation of liver cells with low concentrations of the Ca^{2+} ionophore A23187 also led to increases in PDH_a (Assimacopoulos-Jeannet *et al.*, 1983). Moreover, these hormonal effects on PDH_a were not evident in cells which had been depleted of Ca^{2+} (Assimacopoulos-Jeannet *et al.*, 1983; Oviyasu & Whitton, 1984); however, they could be restored by the subsequent addition of Ca^{2+} (McCormack *et al.*, 1985). There is also no evidence for any changes in the tissue or cell contents of other regulators of the enzymes of the pyruvate dehydrogenase system being brought about by these hormones (Hems *et al.*, 1978; Assimacopoulos-Jeannet *et al.*, 1983), such as ADP/ATP or CoA/acetyl-CoA ratios, pyruvate or H^+ (see Denton *et al.*, 1975).

There is some indirect evidence from studies on intact liver tissue or cells that the 2-oxoglutarate dehydrogenase complex may also be activated by these same hormones (see, e.g., Siess & Wieland, 1978; Sugden *et al.*, 1980; Palmer *et al.*, 1983; Taylor *et al.*, 1983; Häusinger & Sies, 1984; Ochs, 1984; Staddon & McGivan, 1984, 1985). The evidence includes the lowering of the tissue or cell contents of 2-oxoglutarate (and glutamate) when the flux through 2-oxoglutarate dehydrogenase is increased. These effects also appear to be Ca^{2+} -dependent (in the manner discussed above for PDH_a), and Staddon & McGivan (1984, 1985) have reported additive effects of glucagon with vasopressin.

The above indirect evidence, taken together with the findings of the preceding paper (McCormack, 1985b), suggest that in the liver these hormones may, as the result of increasing cytoplasmic $[\text{Ca}^{2+}]$, also cause increases in intramitochondrial $[\text{Ca}^{2+}]$ and so activate these Ca^{2+} -sensitive dehydrogenases. However, many workers have reported that liver mitochondrial Ca^{2+} is decreased by these hormones and have argued that the Ca^{2+} so mobilized accounts for some or all of the increases in cytoplasmic $[\text{Ca}^{2+}]$ (see, e.g., Babcock *et al.*, 1979; Blackmore *et al.*, 1979; Exton, 1981; Williamson *et al.*, 1981; Reinhart *et al.*, 1982b; Whiting & Barritt, 1982; Joseph & Williamson, 1983; Studer & Borle, 1983; Baddams *et al.*, 1983).

The present study uses the techniques described in the preceding paper (McCormack, 1985b), for assaying the Ca^{2+} -sensitive properties of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes in intact rat liver mitochondria, in adopting the more direct

experimental strategy, developed initially for the rat heart by McCormack & Denton (1984), for assessing the effects of hormones on intramitochondrial $[\text{Ca}^{2+}]$. Results of a preliminary study on the effects of adrenaline have been published previously (McCormack, 1985a).

EXPERIMENTAL

Mitochondria were prepared from the livers of male or female (as indicated) fed Wistar rats of 225–275 g body wt., which had either been anaesthetized by the intraperitoneal injection of 0.25 ml of Sagatal (May and Baker, Dagenham, Essex, U.K.) for at least 10 min before being subjected to the treatments indicated in Tables 1, 5, 6 and 7 and Fig. 2, or else decapitated (Tables 2–4, Fig. 1), as described by McCormack (1985a, b), respectively. In the former instance three pieces (approx. 1 g) of liver (from different lobes) were rapidly homogenized; when appropriate, a small piece from a fourth lobe was quickly freeze-clamped (at the temperature of liquid N_2) for subsequent analysis of tissue PDH_a content.

Details of the methods used to incubate mitochondria and of the various techniques used to assay for the Ca^{2+} -sensitive properties of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes within intact rat liver mitochondria are given in the preceding paper (McCormack, 1985b). This includes the method of extraction of mitochondria for the assay of pyruvate dehydrogenase, and also details on the use of Ca-EGTA buffers, the assays for mitochondrial ATP and protein contents, and methods of kinetic and statistical analyses. Liver tissue was extracted and assayed for the amount of PDH_a as described by Hems *et al.* (1978), and total pyruvate dehydrogenase activity was determined after treatment of samples as described in McCormack *et al.* (1982). The latter was unaffected by any of the treatments used in the present study and was in the range 1.6–2.4 units/g wet wt. of tissue or 35–60 munits/mg of mitochondrial protein (where 1 unit catalyses the formation of 1 μmol of acetyl-CoA/min at 30 °C), and results are given as the percentage of the total activity existing as PDH_a . In addition, none of the treatments used affected the yield (25–35%) of mitochondria as assessed by the recovery of total pyruvate dehydrogenase activity. All other relevant conditions and procedures are given in the Figure and Table legends. Note that potassium salts were used unless otherwise stated, that where 10 mM-NaCl was present in incubations 10 mM-KCl was omitted from the buffer (see Fig. 1), and also that in time-course studies involving measurements made in sedimented mitochondria (Tables 2, 4, 5 and 6; Fig. 1), the time of sedimentation (20–25 s) has been included as part of the times of incubation given.

The methods used for loading rat liver mitochondria with ^{45}Ca and the assay of mitochondrial ^{45}Ca content were essentially as described by McCormack & Denton (1984) for rat heart mitochondria, except that the amounts of unlabelled CaCl_2 required to be added to achieve the appropriate degrees of enzyme activation were assessed by measuring 2-oxoglutarate-induced NAD(P)^+ reduction (as described by McCormack, 1985b), rather than 2-oxoglutarate-dependent O_2 uptake.

Sources of all materials used are given in the preceding paper (McCormack, 1985b), except for: adrenaline (BDH Chemicals, Poole, Dorset, U.K.); glucagon (Calbiochem, Bishops Cleeve, Herts, U.K.); pyrophosphate (Sigma,

Table 1. Effects of adrenaline or glucagon alone, or together, on the amount of liver PDH_a in normal fed male and female rats *in vivo*

Anaesthetized rats (see the Experimental section) of the sex indicated were injected intraperitoneally with: 0.3 ml of saline [0.9% (w/v) NaCl] (control) for 15 min; 0.25 mg of adrenaline (tartrate) in 0.3 ml of saline for 2 min; 0.1 mg of glucagon (in 0.1 ml of 0.5 M-NaCl/20 mM-Tris, pH 10.0) for 15 min; or with glucagon (15 min) together with adrenaline for the last 2 min, as indicated, before samples of liver were removed and freeze-clamped for subsequent PDH_a analysis as described in the Experimental section. These times and doses of hormone treatment were found to give optimum effects; the injection of saline alone (control) yielded similar results after 2, 5 or 20 min (instead of 15 min) to those shown. Results are given as means $\pm$ S.E.M. for the numbers of rats in parentheses: * $P \leq 0.05$ and ** $P \leq 0.01$ for the effects of hormone treatment.

Hormone treatment	PDH _a (as % of total activity) in livers from:	
	Female rats	Male rats
None (control)	6.6 $\pm$ 1.2 (6)	12.9 $\pm$ 1.8 (4)
Adrenaline	15.3 $\pm$ 2.8 (4)*	22.6 $\pm$ 3.2 (3)*
Glucagon	14.7 $\pm$ 1.8 (4)**	20.7 $\pm$ 2.5 (3)*
Glucagon with adrenaline	33.8 $\pm$ 5.7 (4)*	46.3 $\pm$ 6.4 (3)**

Poole, Dorset, U.K.); $^{45}\text{CaCl}_2$ (Amersham, Bucks, U.K.); *myo*-inositol 1,4,5-trisphosphate (kindly given by Dr. R. F. Irvine, Department of Biochemistry, AFRC Institute of Animal Physiology, Cambridge, U.K.); compound YS035 [*NN*-bis-(3,4-dimethoxyphenethyl)-*N*-methylamine; kindly given by Dr. R. Deana, Istituto di Chimica Biologica, Università di Padova, Italy] and amiprophosmethyl (kindly given by Bayer A.G., 5090 Leverkusen-Bayerwerk, Germany).

RESULTS AND DISCUSSION

In addition to the studies on the perfused liver or isolated hepatocytes mentioned in the Introduction section, on the activations of pyruvate dehydrogenase by several hormones known to increase liver cytoplasmic $[\text{Ca}^{2+}]$, McCormack (1985a) reported that adrenaline increased liver PDH_a when it was injected into female rats. This observation is repeated in Table 1, which also shows that the injection of glucagon similarly increased PDH_a and that these effects of adrenaline and glucagon *in vivo* were additive. Similar effects were also observed with male rats; however, the amounts of PDH_a always appeared to be higher with the males (Table 1; see McCormack, 1985b). Other workers have reported that there may be sex and age differences in the response of liver cells to adrenaline and glucagon (see, e.g., Morgan *et al.*, 1983; Studer & Borle, 1983; Studer *et al.*, 1984), in particular with regard to the degree to which adrenaline acts through α - or β -adrenergic receptors, and the degree to which this affects the combined response with glucagon. However, it appears that adrenaline does increase cytoplasmic $[\text{Ca}^{2+}]$ in all cases so far examined (Charest *et al.*, 1983; Studer *et al.*, 1984).

The purpose of the present investigation was to try to

Fig. 1. Effects of Ca^{2+} on the amount of PDH_a in rat liver mitochondria from male and female rats, and the determination of appropriate Ca^{2+} -loading conditions

Mitochondria prepared from male (Δ) or female ($\square$, $\blacksquare$) decapitated rats (see the Experimental section) were incubated (at approx. 1 mg of protein/ml) at 30 °C for 5 min in 1 ml of KCl-based medium [125 mM-KCl/20 mM-Tris (pH 7.3)/5 mM-potassium phosphate] which contained 2 mM-2-oxoglutarate, 0.2 mM-L-malate, 1 mM-pyruvate (potassium salt) and appropriate amounts of EGTA-Ca buffers (at 5 mM-EGTA) to give the Ca^{2+} concentrations shown, and in either the absence (Δ , $\square$) or the presence ($\blacksquare$) of 10 mM-NaCl and 1 mM- MgCl_2 . Values shown are means of separate observations made on samples from at least three different preparations of mitochondria. The concentrations of CaCl_2 in mM (and the resultant free $[\text{Ca}^{2+}]$ in nM) at the arrows were: 1, 3.5 ($\approx$ 120); 2, 4 ($\approx$ 230); 3, 4.5 ($\approx$ 500); and 4, 4.8 ($\approx$ 1050). The conditions shown by 1 and 2 refer to the Ca loads 1 and 2 used in Tables 2–4.

ascertain whether these increases in PDH_a (Table 1) were brought about, at least in part, through increases in intramitochondrial $[\text{Ca}^{2+}]$, as consequences of the increases in cytoplasmic $[\text{Ca}^{2+}]$ caused by the hormones, as proposed by Denton & McCormack (1980, 1981). The development of the ability to assay for the Ca^{2+} -sensitive properties of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes within intact rat liver mitochondria (McCormack, 1985b) allowed this hypothesis to be tested by adopting the procedures used by McCormack & Denton (1984) to obtain direct evidence that adrenaline increases intramitochondrial $[\text{Ca}^{2+}]$ in rat heart. This approach involves the rapid preparation of mitochondria from control and treated tissues, followed by their incubation under conditions where the Ca^{2+} -sensitive enzymes can be assayed as indicators of hormone-induced changes in intramitochondrial $[\text{Ca}^{2+}]$. However, to assess the feasibility of this approach it was first of all necessary (as described by McCormack & Denton, 1984) to examine the behaviour of these two enzymes within liver mitochondria which were loaded *in vitro* with Ca^{2+} and then 're-prepared'.

Table 2. Effects of Na⁺, diltiazem and Ca²⁺ on the persistence of increases in PDH_a, caused by the preincubation of liver mitochondria with Ca²⁺, during their 're-isolation' and subsequent incubation with EGTA

Mitochondria were incubated (including substrates) as described for Fig. 1, but at approx. 3 mg of protein/ml and in 4 ml of medium containing EGTA either alone (control) or together with the appropriate [CaCl₂] to give the Ca²⁺ concentrations shown by arrows 1 and 2 in Fig. 1. After 5 min, a large excess (10 vol) of ice-cold sucrose-based isolation medium containing EGTA (McCormack, 1985a) was added and the mitochondria were 're-isolated', with one wash (see McCormack & Denton, 1984). The recovery of mitochondria on re-preparation was over 90%. The 're-isolated' mitochondria were then incubated exactly as in Fig. 1, but with 0.5 mM-EGTA (instead of EGTA-Ca buffers at 5 mM-EGTA) and other additions as indicated below. Results are expressed as in Table 1. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ for the effects of the Ca loads, and † $P \leq 0.05$, †† $P \leq 0.01$, and ††† $P \leq 0.001$ for the effects of Na⁺.

Mitochondrial sample from:	Additions to incubations	Male (M) or female (F) donor	Incubation time (min)	PDH _a (as % of total activity) in mitochondria loaded during the preincubation (see Fig. 1), with		
				no Ca ²⁺ (control)	Ca load 1	Ca load 2
Preincubation	—	M	—	16.4±1.6 (4)	34.6±6.2 (4)*	53.5±7.1 (4)*
		F	—	9.4±1.0 (6)	23.2±3.6 (6)*	35.4±4.2 (4)**
Freshly 're-isolated'	—	M	—	13.9±2.7 (4)	29.8±4.6 (4)*	46.2±3.9 (4)***
		F	—	8.8±0.8 (6)	20.7±3.2 (6)*	30.4±4.6 (4)*
Incubations	None	M	5	18.8±3.1 (4)	38.4±5.1 (4)*	50.6±3.2 (4)***
		F	5	12.1±2.0 (6)	26.9±3.8 (6)**	40.6±6.3 (4)***
			10	11.8±2.1 (3)	29.6±3.2 (3)**	39.8±7.1 (3)*
	10 mM-NaCl	M	5	15.6±1.9 (3)	20.5±1.9 (3)†	26.4±2.5 (3)*††
		F	5	9.9±1.3 (4)	13.1±1.6 (3)†	18.4±2.7 (4)*†
			10	10.2±1.1 (3)	10.9±1.4 (3)††	12.6±1.3 (3)†
	10 mM-NaCl + 300 μM-diltiazem 4 mM-EGTA + 4 mM-CaCl ₂ (resultant free [Ca ²⁺] ≈ 400 nM)	F	5	12.2±1.1 (3)	28.6±4.2 (3)*	40.4±3.7 (3)**
		F	5	50.4±3.9 (4)	49.7±4.8 (4)	47.9±5.4 (4)

Effects of pre-loading rat liver mitochondria with Ca²⁺ on the activities of the pyruvate and 2-oxoglutarate dehydrogenase complexes in subsequently 're-isolated' mitochondria incubated with EGTA and in the absence or presence of Na⁺, diltiazem or CaCl₂

Fig. 1 shows the effects [similar to those described in the preceding paper (McCormack, 1985b)] of increasing extramitochondrial [Ca²⁺] on the amount of PDH_a in rat liver mitochondria incubated with 2-oxoglutarate, malate and pyruvate, and in the absence and presence of Na⁺ and Mg²⁺. The concentrations of Ca²⁺ indicated by the odd- and even-numbered arrows respectively in Fig. 1 were chosen to load the mitochondria with Ca²⁺ as these gave similar degrees of enzyme activation (from the appropriate control amounts of PDH_a) as the hormones did *in vivo* when given alone and together respectively (see Table 1). Essentially similar results were obtained if loading was achieved in the absence or presence of Na⁺ and Mg²⁺ (see Fig. 1); however, only those from the former condition are presented below in Tables 2–4.

Mitochondria that were incubated under the conditions shown by arrows 1 and 2 in Fig. 1 were then 're-isolated' and subsequently incubated as described in Table 2. These Ca²⁺-dependent activations achieved in the preincubations persisted through this 're-preparation', and the following incubations at 30 °C in media containing EGTA, provided that Na⁺ ions were absent. However, these persistent activations could be rapidly diminished if Na⁺, or enough extramitochondrial [Ca²⁺] to cause saturation of the Ca²⁺-dependent activations of the enzyme, was added to the incubations (Table 2).

Moreover, these effects of Na⁺ were blocked by diltiazem, which is known to inhibit the Na⁺-induced egress of Ca²⁺ from heart mitochondria (Vághy *et al.*, 1982; see also McCormack, 1985b), and Na⁺ appeared to take longer to exert its effects with the mitochondria previously subjected to the higher Ca load. The apparent differences in liver PDH_a between males and females were also still evident (Table 2); this was only the case for PDH_a (see McCormack, 1985b), and thus in all other measurements made (below), data obtained from both sexes have been combined. It is worth noting that if no pyruvate was present under the Ca²⁺-loading conditions indicated by arrows 1 and 2 in Fig. 1, then, as noted in McCormack (1985b), no effects of Ca²⁺ on PDH_a could be seen (results not shown). However, if mitochondria from such preincubations were 're-prepared' and then incubated with pyruvate as in Table 2, then the effects of pre-loading with Ca²⁺ became evident, i.e. essentially similar results to those shown in Table 2 could be obtained even though an effect of Ca²⁺ was not evident in the original preincubations. It should also be noted that, when mitochondria were preincubated with EGTA (see Table 2), the additional absence or presence of Na⁺ in this instance resulted in essentially similar values being obtained with the subsequently 're-isolated' mitochondria incubated as in Table 2 (and also as in Table 3, below), suggesting that very little Ca²⁺ was present in the mitochondria as they were originally isolated from untreated rats. Mitochondrial ATP content (6–9 nmol/mg of protein) was not significantly altered by any of the conditions of either preincubation or incubation used in Table 2.

Table 3. Effects of pre-loading liver mitochondria with Ca^{2+} on (a) the rate of 2-oxoglutarate decarboxylation by, and (b) the extent of 2-oxoglutarate-induced NAD(P)H formation in, the subsequently 're-isolated' mitochondria incubated under several different conditions

Mitochondria were preincubated and 're-isolated' as for Table 2, and then incubated [at approx. (a) 0.5 or (b) 1.5 mg of protein/ml and 30 °C] in KCl-based media (without substrates) (see Fig. 1) which contained either (a) 0.5 mM-L-malate or (b) 0.5 mM-malonnate and 0.5 mM- NH_4Cl , together with (in each case) 0.5 mM-EGTA and the additions indicated below. The full assay procedures for both (a) and (b) are given in the preceding paper (McCormack, 1985b). The incubation time given refers to that before 2-oxoglutarate addition; in (a) $^{14}\text{CO}_2$ from 2-oxo[1- ^{14}C]glutarate was collected over the following 2.5 min, and in (b) the extent of NAD(P) $^+$ reduction was measured 3 min after 2-oxoglutarate addition. The extent of reduction induced by 3 mM-2-oxoglutarate (i.e. saturating) was unaffected by the treatments used. Results are expressed and were analysed as for Table 2.

[2-Oxoglutarate] (mM)	Additions to incubation media	Incubation time (min)	(a) Rate of 2-oxoglutarate decarboxylation (nmol/min per mg of protein) by mitochondria pre-loaded with (see Fig. 1):		
			no Ca^{2+} (control)	Ca load 1	Ca load 2
0.05	None	2	0.32 ± 0.05 (3)	1.04 ± 0.13 (3)**	1.46 ± 0.18 (3)**
		5	0.30 ± 0.02 (6)	0.96 ± 0.09 (6)***	1.17 ± 0.18 (3)*
		10	0.29 ± 0.04 (3)	0.85 ± 0.10 (3)**	1.06 ± 0.13 (3)**
	10 mM-NaCl	2	0.31 ± 0.03 (3)	0.88 ± 0.10 (3)**	1.09 ± 0.18 (3)*
		5	0.29 ± 0.02 (6)	0.39 ± 0.05 (6)†††	0.56 ± 0.11 (3)†
		10	0.27 ± 0.03 (3)	0.27 ± 0.02 (3)††	0.34 ± 0.03 (3)††
	10 mM-NaCl + 300 μM -diltiazem 4 mM-EGTA + 4 mM- CaCl_2 (resultant [Ca^{2+}] $\approx$ 400 nM)	5	0.33 ± 0.01 (3)	1.10 ± 0.08 (3)**	1.52 ± 0.14 (3)*
		5	1.66 ± 0.15 (4)	1.62 ± 0.10 (4)	1.57 ± 0.13 (3)
	2.0	5	7.61 ± 1.31 (3)	8.20 ± 1.06 (3)	7.77 ± 0.98 (3)
(b) Extent of NAD(P) $^+$ reduction (as % of extent at saturating [2-oxoglutarate]) in mitochondria pre-loaded as in (a) above					
0.2	None	5	8.4 ± 1.1 (6)	30.6 ± 3.8 (6)**	39.8 ± 4.7 (3)*
	10 mM-NaCl	5	6.4 ± 0.9 (4)	10.1 ± 1.8 (4)††	18.4 ± 5.1 (3)**†
	10 mM-NaCl + 300 μM -diltiazem	5	10.8 ± 0.8 (3)	34.9 ± 4.6 (3)**	42.3 ± 5.1 (3)*
	4 mM-EGTA + 4 mM- CaCl_2	5	49.6 ± 5.8 (3)	46.9 ± 3.6 (3)	50.4 ± 5.2 (3)

Table 3 shows that the activity of 2-oxoglutarate dehydrogenase (assayed at non-saturating [2-oxoglutarate]) within the 're-isolated' and subsequently incubated mitochondria, and as assessed by two different methods [see Table 3 and McCormack (1985b)], appears to parallel the behaviour of PDH $_a$ noted in Table 2. The extents of NAD(P) $^+$ reduction induced by non-saturating concentrations of glutamate, *threo*-D $_3$ -isocitrate or citrate were also enhanced in a similar manner as the result of Ca loading; however, those induced by non-saturating (or saturating) concentrations of β -hydroxybutyrate or L-malate were unaffected (results not shown; see McCormack, 1985b).

Effects of Na^+ and several other agents on the egress of ^{45}Ca from pre-loaded rat liver mitochondria subsequently incubated under a variety of different conditions

McCormack & Denton (1984) described a method whereby rat heart mitochondria could be loaded with suitable amounts of ^{45}Ca (and unlabelled Ca) under apparent conditions of steady-state Ca^{2+} cycling across the mitochondrial inner membrane, and at appropriate points within the activation ranges for Ca^{2+} of the Ca^{2+} -sensitive intramitochondrial enzymes. This was adapted (see the Experimental section) to load rat liver mitochondria in a similar manner while achieving degrees of enzyme activation similar to those indicated by arrows 1 and 2 in Fig. 1.

Table 4 shows that, after their 're-isolation', the results on Ca^{2+} handling by liver mitochondria obtained by monitoring ^{45}Ca egress from these ^{45}Ca -loaded mitochondria matched very closely those obtained (Tables 2 and 3) by monitoring the Ca^{2+} -sensitive enzyme activities under similar conditions. It should be noted, first of all, that very little Ca^{2+} appeared to be lost from the mitochondria at 0 °C, even when Na^+ was present (Table 4), suggesting (as was also found with heart; McCormack & Denton, 1984) that the amounts of Ca^{2+} originally present in liver mitochondria will be maintained during their preparation. Ca^{2+} egress was stimulated in both the absence and the presence of Na^+ as temperature was increased (Table 4); however, at the temperatures used, the effects of Na^+ then became readily evident. Clear effects of Na^+ were also observed (Table 4) under all of the conditions used for assaying the Ca^{2+} -sensitive intramitochondrial dehydrogenases (Tables 2 and 3, and 5–7 below; Fig. 1, and Fig. 2 below). Also consistent with the enzyme data, it is clear (Table 4) that at 10 mM-NaCl and 30 °C the ^{45}Ca took several minutes to leave the mitochondria under these conditions of Ca loading and, as would be expected, effects of Na^+ were still evident for longer incubation times in the samples from the high Ca load (not shown in full). However, it should be noted that Na^+ had caused the enzyme activities to return to control values (Tables 2 and 3) before its effects on ^{45}Ca egress were completed (see below), i.e. Ca^{2+} stimulation appears

Table 4. Effects of various agents and conditions on the egress of ^{45}Ca from pre-loaded rat liver mitochondria subsequently 're-isolated' and incubated in the absence or presence of Na^+

Mitochondria were pre-loaded with ^{45}Ca (see the Experimental section) under similar conditions to those in Table 2, except that pyruvate and EGTA-Ca buffers were absent, and the appropriate $[\text{CaCl}_2]$ to achieve similar degrees of enzyme activation (as in Fig. 1, arrows 1 and 2) was determined as described in the Experimental section; this was approx. 30 and 50 μM for loads 1 and 2 respectively (some EGTA was added with the mitochondrial samples; see McCormack & Denton, 1984), or 70 and 90 μM if Na^+ and Mg^{2+} were present. 'Re-isolated' mitochondria were incubated (approx. 1.5 mg of protein/ml) in KCl-based medium (Fig. 1) which contained 0.5 mM-EGTA and the additions indicated. Results are expressed and were analysed as for Table 2.

Incubation conditions	Other additions	Incubation temp. ($^{\circ}\text{C}$)	Incubation time (min)	^{45}Ca content of 're-isolated' mitochondria [as % of initial (zero-time) content] pre-loaded with:			
				Ca load 1, and incubated with:		Ca load 2, and incubated with:	
				Na^+ absent	10 mM-NaCl	Na^+ absent	10 mM-NaCl
2 mM-2-Oxo-glutarate + 0.2 mM-L-malate	None	0	5	101.1 $\pm$ 1.6 (4)	98.8 $\pm$ 1.0 (4)	100.6 $\pm$ 0.5 (3)	100.9 $\pm$ 1.6 (3)
		20	5	95.5 $\pm$ 3.0 (3)	66.6 $\pm$ 5.8 (3)†	101.5 $\pm$ 1.8 (3)	70.3 $\pm$ 4.1 (3)††
		30	5	87.3 $\pm$ 4.2 (8)	52.5 $\pm$ 3.2 (8)†††	92.2 $\pm$ 2.2 (6)	51.7 $\pm$ 3.7 (6)†††
		37	5	78.4 $\pm$ 3.6 (4)	39.5 $\pm$ 4.1 (4)†††	82.5 $\pm$ 1.8 (3)	45.5 $\pm$ 5.2 (3)†††
	None	30	2	98.7 $\pm$ 3.7 (3)	73.1 $\pm$ 4.5 (3)†	98.9 $\pm$ 1.9 (3)	77.2 $\pm$ 4.5 (3)†
		30	10	74.9 $\pm$ 6.5 (4)	32.2 $\pm$ 2.1 (4)†††	84.0 $\pm$ 2.5 (3)	28.2 $\pm$ 3.6 (3)†††
	300 μM -Diltiazem 1 μM - <i>myo</i> -Inositol 1,4,5-trisphosphate 100 μM -Pyrophosphate 100 μM -Compound YS035 2 μM -Ruthenium Red 100 μM -Amiprophosmethyl A23187 (2 $\mu\text{g}/\text{ml}$)	30	5	96.0 $\pm$ 2.6 (6)	92.3 $\pm$ 2.4 (6)	96.2 $\pm$ 2.8 (6)	97.3 $\pm$ 0.7 (6)
		30	5	84.4 $\pm$ 3.8 (4)	54.0 $\pm$ 4.6 (4)††	88.1 $\pm$ 3.5 (4)	53.6 $\pm$ 4.2 (4)†††
		30	5	87.6 $\pm$ 1.8 (3)	56.5 $\pm$ 2.3 (3)†††	94.1 $\pm$ 1.8 (3)	57.8 $\pm$ 6.8 (3)††
		30	5	90.9 $\pm$ 1.5 (3)	58.1 $\pm$ 4.8 (3)††	93.9 $\pm$ 0.9 (3)	58.7 $\pm$ 3.5 (3)†††
		30	5	89.1 $\pm$ 2.5 (3)	50.5 $\pm$ 4.1 (3)††	89.6 $\pm$ 2.5 (3)	49.7 $\pm$ 4.5 (3)††
		30	5	92.3 $\pm$ 1.6 (3)	56.2 $\pm$ 4.9 (3)††	92.2 $\pm$ 1.8 (3)	55.6 $\pm$ 6.6 (3)††
		30	5	2.1 $\pm$ 0.3 (8)	3.0 $\pm$ 6 (8)	2.9 $\pm$ 0.2 (6)	3.2 $\pm$ 0.5 (6)
No substrate	None	30	5	72.2 $\pm$ 5.2 (4)	47.1 $\pm$ 3.9 (4)††	80.2 $\pm$ 4.1 (4)	44.3 $\pm$ 2.9 (4)†††
0.5 mM-L-Malate	None	30	5	85.3 $\pm$ 3.2 (6)	46.0 $\pm$ 4.8 (6)†††	86.3 $\pm$ 2.8 (6)	50.3 $\pm$ 3.1 (6)†††
0.5 mM-Malonate	0.5 mM- NH_4Cl	30	5	68.8 $\pm$ 4.9 (5)	32.3 $\pm$ 4.6 (5)†††	76.6 $\pm$ 3.8 (4)	40.9 $\pm$ 4.1 (4)†††
	1 mM-MgATP	30	5	83.5 $\pm$ 3.8 (4)	50.4 $\pm$ 3.2 (4)†††	90.4 $\pm$ 1.8 (3)	48.6 $\pm$ 3.9 (3)†††

to be no longer evident when there is still a considerable amount of Ca associated with the mitochondria (see also McCormack & Denton, 1984). The K_m values for these effects of Na^+ in experiments of the type performed in Table 4 (results not shown) were always found to be approx. 1–2 mM, and 10 mM was very close to saturating. Li^+ ions also stimulated ^{45}Ca egress, but with a K_m of approx. 5–8 mM (results not shown).

Liver mitochondria were originally thought not to possess the capacity for Na^+ -dependent Ca^{2+} egress (see, e.g., Carafoli, 1979; Nicholls & Crompton, 1980). However, more recently other workers have also demonstrated this capacity in liver mitochondria (Haworth *et al.*, 1980; Heffron & Harris, 1981; Goldstone & Crompton, 1982; Nedergaard, 1984). Nevertheless, the degree of stimulation of efflux induced by Na^+ in the present study appears to be even more marked than in these others, and it is suggested that this may have been a consequence of using Ca loads which were much lower, though perhaps more physiological, in the present work than in those earlier studies, i.e. the magnitude of the Na^+ -independent pathway(s) may become relatively greater at higher Ca loads. This may also have been the reason behind the lower K_m values for the effects of Na^+

in the present paper, compared with those previous studies.

The values of ^{45}Ca content (as % of original content) under the various conditions of Table 4 were actually very similar for the two Ca loads used even though there was obviously more Ca present at the higher load. This observation suggests that both the Na^+ -independent and Na^+ -dependent egress pathways for Ca^{2+} were working faster at the higher load, and is thus in agreement with the proposal (McCormack, 1985b) that, at mitochondrial Ca contents within the range to which the intramitochondrial enzymes exhibit Ca^{2+} -sensitivity, the egress pathways for Ca^{2+} are not saturated.

The effects of diltiazem in inhibiting Na^+ -induced Ca^{2+} egress from liver mitochondria are clearly shown in Table 4, although, in addition, there was an indication that this drug may also inhibit the Na^+ -independent egress to some extent as well. Ruthenium Red did not appear to affect Ca^{2+} egress (with or without Na^+ ; Table 4), suggesting that the pathways concerned do not simply involve reversal of the Ca^{2+} -uptake pathway, as the latter is potently inhibited by this compound (Moore, 1971; Vasington *et al.*, 1972). The Ca^{2+} ionophore A23187 appeared to deplete the mitochondria completely of Ca

Table 5. Effects of adrenaline and glucagon, alone or together *in vivo*, on the amount of PDH_a in subsequently prepared rat liver mitochondria incubated under several different conditions

Mitochondria were prepared (as given by McCormack, 1985a) from rats treated with hormones as detailed for Table 1, and then incubated as described for Fig. 1 (with substrates), except that 0.5 mM-EGTA (instead of EGTA-Ca buffers) was present with the additions indicated below. Results are expressed and were analysed as for Table 2 (except that * refers to the effects of hormone treatment).

Additions or changes to incubations	Male (M) or female (F) donor	Incubation time (min)	PDH _a (as % of total activity) in mitochondria prepared from rats injected with:				(n)
			Saline (control)	Adrenaline	Glucagon	Glucagon and adrenaline	
(Freshly isolated mitochondria)	M	(Unincubated)	10.4 ± 1.5	20.3 ± 2.3*	21.4 ± 2.4*	39.8 ± 4.5**	(3)
	F		5.8 ± 1.3	14.8 ± 2.8*	13.0 ± 2.8*	31.8 ± 5.0**	(4)
None	M	5	15.8 ± 2.0	29.4 ± 2.8*	31.6 ± 3.5*	40.0 ± 4.6**	(3)
	F	5	12.4 ± 2.3	20.5 ± 2.4*	20.2 ± 2.7*	27.8 ± 3.4**	(7)
	F	10	13.0 ± 1.4	25.4 ± 3.6*	24.4 ± 3.7*	29.0 ± 5.1*	(5)
10 mM-NaCl	M	5	15.2 ± 1.8	17.3 ± 2.8†	16.2 ± 2.5†	23.1 ± 2.8†	(3)
	F	5	13.2 ± 1.6	13.7 ± 1.2†	16.1 ± 2.7	20.2 ± 3.9	(6)
	F	10	15.1 ± 1.6	17.1 ± 3.5	17.4 ± 2.7	18.2 ± 3.1	(5)
300 µM-Diltiazem	F	5	10.9 ± 1.1	17.2 ± 1.7*	17.6 ± 1.9*	26.2 ± 1.9***	(4)
300 µM-Diltiazem + 10 mM-NaCl	F	5	13.2 ± 1.9	23.1 ± 2.9*	23.7 ± 3.3*	33.3 ± 4.2**	(6)
No oxoglutarate	F	5	21.9 ± 2.6	39.2 ± 4.6*	42.9 ± 2.2**	57.2 ± 5.9**	(3)
No oxoglutarate + 10 mM-NaCl	F	5	24.1 ± 1.8	32.1 ± 8.0	34.2 ± 3.4	40.8 ± 6.2	(3)
At pH 7.6	F	5	18.5 ± 1.4	38.2 ± 5.7*	32.5 ± 2.9*	39.9 ± 2.3**	(3)
At pH 7.6 + 10 mM-NaCl	F	5	20.5 ± 1.5	25.3 ± 5.8	23.3 ± 2.4	24.5 ± 0.9††	(3)
1 mM-EGTA + 1 mM-CaCl ₂ (final [Ca ²⁺] ≈ 110 nM)	F	5	24.8 ± 1.0	28.9 ± 1.2	27.3 ± 1.2	36.5 ± 3.3*	(3)
4 mM-EGTA + 4 mM-CaCl ₂ (≈ 400 nM)	F	5	49.9 ± 4.3	51.2 ± 5.8	44.6 ± 5.6	46.5 ± 5.5	(4)

(Table 4), such that the amount of ^{45}Ca in the volume occupied by the mitochondrial pellet obtained was essentially the same as that in a similar volume of the resulting supernatant (results not shown). It should be noted that Na^+ could not achieve such an extent of depletion (results not shown); approx. 20% (load 1) or 10% (load 2) of the ^{45}Ca remained in the mitochondria after effects of Na^+ , with respect to time, were no longer evident. The reason(s) for this are unknown (but see also McCormack & Denton, 1984).

Several other compounds have been reported to affect Ca^{2+} egress from rat liver mitochondria. In particular, amiprophosphomethyl (Hertel *et al.*, 1981) and pyrophosphate (Vercesi & Lehninger, 1984) were reported to stimulate, and compound YS035 (Deana *et al.*, 1984) to inhibit, this process. In addition, *myo*-inositol 1,4,5-trisphosphate was shown to cause Ca^{2+} egress from a crude mitochondrial fraction by Joseph *et al.* (1984), although it was pointed out that the source of the Ca^{2+} in this instance was likely to be a contaminating non-mitochondrial fraction. No significant effects (with or without Na^+) of any of these compounds were observed on ^{45}Ca egress under the conditions indicated in Table 4; neither were any observed at 1 mM- or 25 mM-NaCl, or at concentrations of the above proposed effectors either 5-fold less or 5-fold greater than those given (results not shown). In addition, no effects on ^{45}Ca uptake were found, or on Ca^{2+} uptake or egress as assessed by monitoring the Ca^{2+} -sensitive intramitochondrial dehydrogenases (results not shown). Possible reasons behind these discrepancies include the much lower, though perhaps more physiological (see McCormack, 1985b), loads of Ca imposed on the

mitochondria in the present study (compared with those above), or perhaps the inadvertent use of Na^+ salts of the above proposed effectors, or perhaps, as in Joseph *et al.* (1984), mitochondrial fractions used in these other studies being contaminated with other Ca-containing entities.

Effects of the administration *in vivo* of hormones that are known to increase liver cytoplasmic $[\text{Ca}^{2+}]$ on the activities of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes in subsequently prepared mitochondria incubated under a variety of different conditions

Table 5 shows that the increases in liver PDH_a brought about by the injections of male or female rats with adrenaline and/or glucagon (Table 1), persisted through the preparation of mitochondria and their subsequent incubations in Na^+ -free KCl-based media containing EGTA at 30 °C. The comparison of the results presented in Table 5 with those presented in Table 2 (for PDH_a in the mitochondria Ca-loaded *in vitro*) provides strong evidence that these increases in PDH_a caused by the hormones *in vivo* were the results of their causing increases in intramitochondrial $[\text{Ca}^{2+}]$. Some additional incubation conditions are presented in Table 5; these too gave similar results (not shown) in experiments of the type performed in Table 2. The incubations in the absence of 2-oxoglutarate and at pH 7.6 represent attempts to make the differences caused by hormone treatment or Na^+ addition more marked (Table 5); the former appeared to work better, as in the latter the effects of Na^+ were not so marked. This was probably because under these

Table 6. ATP content of mitochondria incubated under some of the conditions of Table 5

Portions of some of the samples from female donors (as indicated) were analysed for ATP content. Results are expressed (and were analysed) as for Table 5.

Additions to incubations	Incubation time (min)	ATP content (as nmol/mg of protein) of mitochondria prepared from rats injected with:				(n)
		Saline (control)	Adrenaline	Glucagon	Glucagon + adrenaline	
None	5	8.9 ± 1.2	9.8 ± 0.5	9.5 ± 0.4	12.3 ± 1.1	(6)
	10	9.5 ± 1.3	10.8 ± 1.0	10.9 ± 0.5	14.0 ± 2.0	(4)
10 mM-NaCl	5	10.5 ± 0.8	11.3 ± 0.2	11.4 ± 1.1	13.4 ± 2.0	(6)
10 mM-NaCl + 300 μ M-diltiazem	5	10.5 ± 1.1	11.1 ± 1.3	10.6 ± 1.3	14.3 ± 1.3	(6)
4 mM-EGTA + 4 mM-CaCl ₂	5	9.8 ± 1.5	10.2 ± 1.1	8.8 ± 0.5	11.0 ± 0.8	(4)

conditions the mitochondrial ATP content fell to about 25% of that in the presence of 2-oxoglutarate; thus PDH_a kinase may perhaps have lacked sufficient ATP to bring about the inactivation of PDH_a back down to control values. Table 6 shows that otherwise the treatments used in Table 5 did not markedly affect mitochondrial ATP content, with the possible exception that, in mitochondria from rats injected with both hormones, there may have been a slight (about 20%), but not significant, increase. It should be noted that other workers have found increased mitochondrial ATP contents and ATP/ADP ratios in liver mitochondria from rats injected with glucagon alone [though generally for longer time periods than in the present study; see, e.g., Halestrap *et al.* (1983) for a review]. At any rate, mitochondrial ATP content appears either not to change or else to change in the direction opposite to that required to result in increases in amounts of PDH_a.

The ability to assay for the Ca²⁺-sensitive properties of a second intramitochondrial enzyme in isolated liver mitochondria (McCormack, 1985b) allowed strong evidence to be obtained (Table 7, Fig. 2) in support of the proposal that persistent increases in PDH_a owing to hormone treatment (Table 5) were the result of increases in intramitochondrial [Ca²⁺]. The effects of hormone pretreatment on the activity of the 2-oxoglutarate dehydrogenase complex as assessed by the rate of decarboxylation of 2-oxoglutarate are shown in Table 7. A comparison of these data with, first of all, that in Table 3 from the Ca²⁺-loading experiments *in vitro*, and secondly, with that in Table 5 for the effects on PDH_a of hormone treatment *in vivo*, strongly suggests that this enzyme is also activated by these hormone treatments and in a manner very similar to that evident for pyruvate dehydrogenase activation. This is the case both in terms of the relative magnitude of the effects on each enzyme and also in terms of the evidence that the effects are due to increases in intramitochondrial [Ca²⁺]. It should be noted that changes in pH lead to opposite changes in the activities of these two enzymes (see e.g., Tables 5 and 7), meaning that the effects of Na⁺ or hormone treatment noted are very unlikely to have been caused by changes in intramitochondrial pH. Again some additional incubation conditions are presented in Table 7; these too could be matched by similar experiments of the type performed for Table 3.

The assessment of the activity of the 2-oxoglutarate

dehydrogenase complex within isolated liver mitochondria by monitoring 2-oxoglutarate-induced NAD(P)H formation (McCormack, 1985b) afforded a particularly striking demonstration of the effects of these hormone pretreatments, and of the effects of Na⁺ or saturating Ca²⁺ in diminishing them (Fig. 2). These could be observed both in the absence of ADP and also in the presence of amounts of MgATP which resulted in concentrations of ADP sufficient to produce (steady-state) 'State 3.5' (Chance & Williams, 1956) respiration conditions (see McCormack, 1985b). However, no effects of hormones were observed on O₂ uptake, induced by either non-saturating or saturating concentrations of 2-oxoglutarate, achieved in either the absence or the presence of ADP or MgATP (results not shown; see McCormack, 1985b). It should also be noted that the original degree of apparent NAD(P)⁺ reduction at the start of the oxidative preincubations (results not shown, but see McCormack, 1985b), which was presumably due to endogenous substrates, was, although very variable, often much higher in mitochondria prepared from hormone-treated rats compared with those from untreated rats. This could have been due to the presence of higher intramitochondrial [Ca²⁺] together with a 'Ca²⁺-sensitive' substrate in the former instance. However, after the preincubations, i.e. from the times shown in Fig. 2, the amounts of maximal NAD(P)⁺ reduction (per mg of protein) that could be attained were very similar in each case. Fig. 2 also shows that, under the conditions used in Table 5 for the PDH_a measurements, the amounts of mitochondrial NAD(P)H would have been, if anything, slightly higher in those prepared from hormone-treated rats (compared with controls). Thus this effector of pyruvate dehydrogenase activity (see Denton *et al.*, 1975) also appeared to change in the direction opposite to that whereby it could lead to increases in PDH_a. The reductions of NAD(P)⁺ induced by non-saturating concentrations of citrate or glutamate were also found to be enhanced by these hormonal treatments; however, those induced by non-saturating concentrations of β -hydroxybutyrate were unaffected (results not shown).

General discussion and conclusions

The results of the present paper are strong evidence that adrenaline and glucagon each activate both the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes in rat liver by causing increases in intramitochon-

Table 7. Effects of adrenaline and glucagon, alone or together *in vivo*, on the rate of 2-oxoglutarate decarboxylation by subsequently prepared rat liver mitochondria incubated under several different conditions

Mitochondria were prepared as for Table 5 and then incubated as in Table 3(a). The [2-oxoglutarate] was 50 μM , except where indicated†. Results are expressed and were analysed as for Table 5.

Additions or changes to incubations	Incubation time (min)	Rate of 2-oxoglutarate decarboxylation (nmol/min per mg of protein) by mitochondria prepared from rats injected with:				(n)
		Saline (control)	Adrenaline	Glucagon	Glucagon + adrenaline	
None	5	0.56 ± 0.07	0.90 ± 0.12*	1.14 ± 0.17*	1.66 ± 0.28*	(5)
10 mM-NaCl	5	0.51 ± 0.10	0.51 ± 0.07†	0.54 ± 0.04†	0.76 ± 0.13†	(4)
0.5 mM-L-Malate	2	0.87 ± 0.11	1.33 ± 0.13*	1.42 ± 0.15*	2.11 ± 0.31**	(5)
	5	0.63 ± 0.07	0.90 ± 0.08*	1.03 ± 0.12*	1.64 ± 0.20**	(9)
	10	0.68 ± 0.05	0.85 ± 0.06*	1.16 ± 0.12**	1.72 ± 0.31*	(5)
0.5 mM-L-Malate, 10 mM-NaCl	2	0.87 ± 0.10	1.02 ± 0.12	1.10 ± 0.12	1.74 ± 0.30*	(4)
	5	0.59 ± 0.06	0.52 ± 0.08††	0.53 ± 0.10††	0.79 ± 0.14††	(9)
	10	0.56 ± 0.04	0.49 ± 0.09†	0.56 ± 0.10††	0.67 ± 0.13†	(5)
0.5 mM-L-Malate, 300 μM -diltiazem	5	0.71 ± 0.05	1.42 ± 0.21*	1.25 ± 0.17*	1.96 ± 0.32*	(4)
0.5 mM-L-Malate, 300 μM -diltiazem, 10 mM-NaCl	5	0.71 ± 0.07	1.23 ± 0.14**	1.22 ± 0.17*	1.95 ± 0.32**	(7)
1 mM-Succinate	5	0.51 ± 0.08	1.15 ± 0.18*	1.07 ± 0.15*	1.26 ± 0.18**	(5)
1 mM-Succinate, 10 mM-NaCl	5	0.37 ± 0.05	0.42 ± 0.09†	0.42 ± 0.08††	0.45 ± 0.05†	(4)
0.5 mM-Malate	5	1.05 ± 0.09	2.27 ± 0.16***	2.31 ± 0.25**	4.92 ± 0.44**	(4)
0.5 mM-Malate, 10 mM-NaCl	5	0.92 ± 0.11	1.23 ± 0.12††	1.09 ± 0.13†	2.12 ± 0.30††	(3)
At pH 7.0, 0.5 mM-L-malate	5	1.11 ± 0.08	2.16 ± 0.21**	2.08 ± 0.17**	4.01 ± 0.26***	(3)
At pH 7.0, 0.5 mM-L-malate, 10 mM-NaCl	5	1.05 ± 0.10	1.15 ± 0.13†	1.22 ± 0.15†	1.93 ± 0.24††	(3)
0.5 mM-L-Malate, 1 mM-EGTA, 1 mM- CaCl_2 (110 nM- Ca^{2+})	5	1.26 ± 0.17	1.41 ± 0.28	1.28 ± 0.09	1.85 ± 0.34	(3)
0.5 mM-L-Malate, 4 mM-EGTA, 4 mM- CaCl_2 (400 nM- Ca^{2+})	5	2.61 ± 0.15	2.42 ± 0.22	2.37 ± 0.14	2.38 ± 0.10	(7)
0.5 mM-L-Malate, ‡2 mM-2-oxoglutarate as substrate	5	7.75 ± 0.47	6.88 ± 0.40	7.42 ± 0.66	7.85 ± 0.41	(6)

drial $[\text{Ca}^{2+}]$, and, moreover, that their effects, both on these enzymes and on intramitochondrial $[\text{Ca}^{2+}]$, are additive. Presumably the third Ca^{2+} -sensitive intramitochondrial enzyme, NAD^+ -isocitrate dehydrogenase (Denton *et al.*, 1978), will also be activated in these circumstances, and some evidence for this was obtained (see the preceding paragraph); however, the Ca^{2+} -sensitive properties of this enzyme are more difficult to assay for in liver mitochondria than are those of the other two enzymes (see McCormack, 1985b). These increases in intramitochondrial $[\text{Ca}^{2+}]$ are likely to form important parts of the mechanisms whereby these hormones stimulate oxidative metabolism in the liver. In agreement with this, these stimulations of oxidative metabolism (see, e.g., Yamazaki *et al.*, 1980; Yip & Lardy, 1981; Reinhart *et al.*, 1982a; Binet & Claret, 1983; Blackmore *et al.*, 1983; Halestrap *et al.*, 1983) appear to be accompanied by increases in mitochondrial NADH/NAD^+ ratios (see, e.g., Sugano *et al.*, 1980; Balaban & Blum, 1982; Blackmore *et al.*, 1983).

Another major conclusion of this, and also the preceding (McCormack, 1985b), paper is that the concentration of Ca^{2+} in the matrix of liver mitochondria is most likely to exist within the range to which these enzymes show Ca^{2+} -sensitivity under normal physiological conditions. Indeed the lack of any significant effect of Na^+ on the mitochondria from untreated animals indicates

that in unstimulated liver cells the intramitochondrial $[\text{Ca}^{2+}]$ exists below, or at the lowest end of, the range to which the enzymes show Ca^{2+} -sensitivity, i.e. suggesting $\leq$ approx. 0.1 μM -free Ca^{2+} (see Denton & McCormack, 1980, 1981). However, it should be noted that some of the data obtained from the mitochondria of untreated rats, and in particular that from Table 7 and a comparison between the 2 and 5 min values obtained for 2-oxoglutarate dehydrogenase in the presence of malate (with or without Na^+), is perhaps indicative of there having been some (albeit small) degree of Ca^{2+} -dependent enzyme activation in the unstimulated liver cells in these instances.

There are now two important mammalian tissues [rat heart (McCormack & Denton, 1984) and liver (the present paper)] wherein hormones binding to the cell membrane appear to cause increases in intramitochondrial $[\text{Ca}^{2+}]$ and hence activation of these enzymes. However, these enzymes are known to exhibit Ca^{2+} -sensitivity in extracts of mitochondria (or the tissues themselves) from all vertebrate tissues so far examined, including the human heart, but not in extracts from invertebrates (see McCormack & Denton, 1981). Therefore Denton & McCormack (1980, 1981) have proposed that the control of these key enzymes' activities through hormones causing changes in intramitochondrial $[\text{Ca}^{2+}]$ may be a widespread phenomenon. As has been discussed previously (McCormack & Denton, 1984), the simplest mechanism

Fig. 2. Effects of the administration *in vivo* of adrenaline and glucagon, alone or together, on NAD(P)H formation by 2-oxoglutarate in subsequently prepared and incubated rat liver mitochondria

Mitochondria prepared from rats injected with 1, saline, 2, adrenaline, 3, glucagon, or 4, glucagon with adrenaline, as described for Table 1, were preincubated [to oxidize endogenous NAD(P)H] as described for Fig. 1 in 2 ml of KCl-based medium which contained 0.5 mM-EGTA, 0.5 mM-malonate and either (a) 0.5 mM-NH₄Cl, or (b) 1 mM-MgATP, and, for (ii) in each case, the additional presence of 10 mM-NaCl. Preincubation, up to the start of the traces shown, were for 5 min, except for the cases in (ii) (broken lines), which were for 10 min. See the preceding paper (McCormack, 1985b) for full details of the method and procedure used. NAD(P)⁺ reduction was then monitored (as shown, and with a dual-wavelength spectrophotometer) after the additions, at the arrows, of: (a) a, 100 μM-2-oxoglutarate; b, 2 mM-2-oxoglutarate; and c, either (continuous lines) 0.5 μg of rotenone or (broken line) 3.5 mM-EGTA plus 3.5 mM-CaCl₂ (resultant free [Ca²⁺] ≈ 340 nM); and (b) a, 300 μM-2-oxoglutarate; b, 2 mM-2-oxoglutarate; and c, either (continuous lines) 0.5 μg of rotenone or (broken line) 5 mM-EGTA plus 5 mM-CaCl₂ (≈ 500 nM). The 100% reduction refers to that in the presence of rotenone, and 0% reduction to that at the indicated start of each trace, in each case. This was done to allow direct comparison of data, as slightly different yields of mitochondria (in terms of mg of protein/ml of suspension), and hence extents of reduction that could be achieved, were obtained in the different preparations. However, the treatments used did not affect the extent of maximal reduction that could be achieved per mg of mitochondrial protein. A typical experiment is shown; similar data were obtained in at least two other experiments.

by which such changes in intramitochondrial [Ca²⁺] could be brought about would involve them occurring in parallel with, though perhaps secondary to, changes in cytoplasmic [Ca²⁺]. There are many instances in many mammalian tissues and cells where hormones and other extracellular agents are known to affect cytoplasmic processes by causing changes in cytoplasmic [Ca²⁺] (see, e.g., Rasmussen & Barrett, 1984).

In the liver it is known that adrenaline and glucagon (and several other hormones) cause increases in cytoplasmic [Ca²⁺] (see, e.g., Charest *et al.*, 1983; Berthon *et al.*, 1984; Thomas *et al.*, 1984; Studer *et al.*, 1984). Using Quin 2, Charest *et al.* (1983) showed that adrenaline and glucagon each increased hepatocyte cytoplasmic [Ca²⁺] by about the same magnitude, from about 0.2 to 0.6 μM. Such values for increases in extramitochondrial [Ca²⁺] agree very well with those required to achieve an approximate Ca²⁺-dependent doubling (from control values) of both the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complex activities in isolated liver mitochondria incubated with physiological concentrations of Na⁺ and Mg²⁺ (McCormack, 1985b); this would correspond to about 50% of the maximal

Ca²⁺-dependent effects on these enzymes. However, it is not known if cytoplasmic [Ca²⁺] increases further if both adrenaline and glucagon are added together, perhaps because 0.6 μM-Ca²⁺ is towards the top end of the range of [Ca²⁺] to which Quin 2 can usefully respond. Nevertheless it would appear that the cytoplasmic Ca²⁺-dependent activation of phosphorylase, which is thought to be one of the major metabolic responses of liver cells to these hormones, is fully saturated by this concentration of Ca²⁺ (see, e.g., Charest *et al.*, 1983). It is of interest that Morgan *et al.* (1983) have reported that such a combination of hormones appears to result in Ca accumulation into liver cells, and in particular into a mitochondrial fraction. The results of Tables 5 and 7 suggest, however, that even with both hormones present, the intramitochondrial Ca²⁺-sensitive enzymes may not yet be fully saturated with Ca²⁺.

Another interesting possibility which should be taken into account in this context is that changes in matrix [Ca²⁺] could be achieved independently of changes in cytoplasmic [Ca²⁺] by, for example, hormones in some way causing changes in the activities of components of the Ca²⁺-transport system of the mitochondrial inner

membrane. Indeed there are already reports that α -adrenergic agonists stimulate Ca^{2+} uptake by liver (Taylor *et al.*, 1980) and heart (Kessar & Crompton, 1981) mitochondria and that, in the former, β -adrenergic agonists stimulate Na^+ -dependent Ca^{2+} egress (Goldstone & Crompton, 1982; Goldstone *et al.*, 1983). In addition, Coll *et al.* (1982) have suggested that α -adrenergic agonists may alter the affinity of intramitochondrial Ca ligands for Ca^{2+} .

It is well known that these hormones initially bring about increases in liver cytoplasmic $[\text{Ca}^{2+}]$ by causing the mobilization of Ca from intracellular stores (see, e.g., Charest *et al.*, 1983; Thomas *et al.*, 1984; Berthon *et al.*, 1984). The present results are in full accord with the proposals that these hormones do this by stimulating the production of *myo*-inositol 1,4,5-trisphosphate in liver cells, which then causes Ca^{2+} release from pools in an endoplasmic-reticulum fraction (Burgess *et al.*, 1984; Joseph *et al.*, 1984; Dawson & Irvine, 1984; Berridge & Irvine, 1984). However, they disagree with the proposals that these hormones increase cytoplasmic $[\text{Ca}^{2+}]$ by eliciting Ca^{2+} release from liver mitochondria by an unknown mechanism (see, e.g., Babcock *et al.*, 1979; Blackmore *et al.*, 1979; Exton, 1981; Williamson *et al.*, 1981; Reinhart *et al.*, 1982b; Whiting & Barritt, 1982; Baddams *et al.*, 1983; Studer & Borle, 1983; Joseph & Williamson, 1983). The latter proposals were largely based on observations of total mitochondrial fraction Ca content after tissue or cell disruption, or on attempts to assess uncoupler-releasable Ca^{2+} from intact cells. Subsequently, however, Kleineke & Söling (1985) have shown that the application of the latter technique to the perfused liver, rather than to intact cells, yielded results which suggest that there is insufficient Ca attributable to mitochondria to bring about the known increases in cytoplasmic $[\text{Ca}^{2+}]$. These authors also argue that previous misconceptions in this instance may have resulted from cells and mitochondria becoming loaded with supraphysiological amounts of Ca during hepatocyte preparations. The observations on liver mitochondrial Ca content *in situ* by using X-ray probe microanalysis (Somlyo *et al.*, 1985) also suggest that there is insufficient Ca located in liver mitochondria to account for the hormone-induced increases in cytoplasmic $[\text{Ca}^{2+}]$; moreover they also showed that there are large pools of Ca in the endoplasmic reticulum. They proposed a mitochondrial Ca content of about 1.1 nmol/mg of protein in normal, untreated, liver cells. This value is lower than all of those obtained in the studies mentioned above which estimated this parameter in disrupted tissues or cells, even after hormone treatment. One possible source of error in these observations is that the mitochondrial fractions isolated were contaminated with the hormonally sensitive reticulum fraction (see, e.g., Katz *et al.*, 1983), which, of course, would have lost Ca after hormone treatment. Indeed Joseph *et al.* (1984) observed *myo*-inositol 1,4,5-trisphosphate-induced release of Ca^{2+} from a crude liver mitochondrial fraction. A suggestion from these studies (Katz *et al.*, 1983; Joseph *et al.*, 1984) is that there may be a hormonally sensitive reticulum pool of Ca which is intimately associated with mitochondria; this could thus allow the rapid transfer of Ca^{2+} to the mitochondria. It is of interest that, when ^{45}Ca is used to assess the Ca content of liver mitochondrial fractions, an increase in this parameter in response to these hormones always seems to be observed (see, e.g., Foden & Randle, 1978;

Poggioli *et al.*, 1980; Berthon *et al.*, 1981; Shears & Kirk, 1984). The present study should have the advantage of avoiding all such contamination problems, because exclusively intramitochondrial Ca^{2+} -sensitive parameters were measured, although there does remain the possibility of hormones causing changes in Ca-binding affinities within the mitochondria (Coll *et al.*, 1982).

I acknowledge the help and advice given to me by Dr. R. M. Denton and Dr. A. P. Halestrap (Department of Biochemistry, University of Bristol) and thank the former for critically reading my manuscript. I also thank Mrs. C. Netto for her excellent technical assistance, Professor J. B. Chappell (Department of Biochemistry, University of Bristol) for the use of his dual-wavelength spectrophotometer, and Dr. P. J. England (Smith, Kline and French Research, Welwyn, Herts.) for making available computer programs. These studies were supported by a project grant from the Medical Research Council, together with some additional funds kindly made available from a group grant awarded to Dr. R. M. Denton by the British Diabetic Association.

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