Studies on the interactions of Ca^{2+} and pyruvate in the regulation of rat heart pyruvate dehydrogenase activity

Effects of starvation and diabetes

James G. McCORMACK, Nigel J. EDGELL and Richard M. DENTON

Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

(Received 7 September 1981/Accepted 3 November 1981)

1. Previous studies showed that the activation of pyruvate dehydrogenase within intact rat heart mitochondria on addition of pyruvate is much diminished in mitochondria from starved or diabetic animals [see Kerbey, Randle, Cooper, Whitehouse, Pask & Denton (1976) *Biochem. J.* 154, 327–348]. In the present study, diminished responses to added Ca^{2+} and ADP were also found in these mitochondria. 2. Starvation or diabetes did not affect the mitochondrial respiratory control ratio or the ATP content. Moreover, starvation and diabetes did not alter the response of the intramitochondrial Ca^{2+} -sensitive enzyme, 2-oxoglutarate dehydrogenase, to changes in the extramitochondrial concentration of Ca^{2+} and 2-oxoglutarate, thus indicating that there were no appreciable changes in the distribution of Ca^{2+} and H^+ across the mitochondrial inner membrane. 3. Pyruvate, Ca^{2+} and ADP were found to have synergistic effects on pyruvate dehydrogenase activity, particularly in mitochondria from starved and diabetic rats. 4. The results suggest that the effects of diabetes and starvation on pyruvate dehydrogenase are not brought about by changes in the distribution of these effectors across the mitochondrial inner membrane or by changes in the intrinsic sensitivity of the kinase or phosphatase of the pyruvate dehydrogenase system to pyruvate, Ca^{2+} or ADP; rather it is probable that there is an increase in the maximum activity of kinase relative to that of the phosphatase. 6. The results also lend further support to the hypothesis that adrenaline may bring about the activation of pyruvate dehydrogenase in the rat heart by an increase in the intramitochondrial concentration of Ca^{2+} .

The amount of mammalian pyruvate dehydrogenase complex present in its active, non-phosphorylated form (PDH_a) is determined by the relative activities of its interconverting phosphatase and kinase. The activity of each of these enzymes is controlled by various effectors. The phosphatase is activated by Mg^{2+} ($k_{0.5} \approx 1 \text{ mM}$) and by Ca^{2+} ($k_{0.5} \approx 1 \mu\text{M}$). The MgATP -dependent kinase is inhibited by pyruvate, ADP and perhaps Ca^{2+} , and activated by increases in the concentration ratios of NADH/NAD^+ and acetyl-CoA/CoA (for reviews, see: Denton *et al.*, 1975; Denton & Hughes, 1978; Randle *et al.*, 1979). The whole of the pyruvate dehydrogenase system is located exclusively within the mitochondrial matrix (see Denton *et al.*, 1975). In experiments with intact mitochondrial preparations, manipulations of the intramitochondrial

concentrations of all the above effectors have been shown to result in the appropriate changes in amounts of PDH_a (see above references).

In particular, increases in the concentration of pyruvate in the medium have been shown to result in increases in the amount of PDH_a in both intact heart mitochondria and perfused heart preparations from normal fed rats (Kerbey *et al.*, 1976). However, in the same preparations from starved or diabetic rats the extent of the increases in the amount of PDH_a is much diminished (Kerbey *et al.*, 1976). Similar diminished responses to pyruvate have also been observed in preparations of other mammalian tissues, including adipose tissue and mammary gland (Denton, 1975; Baxter *et al.*, 1979). These observations and further studies on pyruvate dehydrogenase kinase activity in tissue and mitochondrial extracts have led to the proposal that starvation and diabetes lead to a loss of the inhibition of the kinase by pyruvate (Baxter & Coore, 1978; Hutson *et al.*, 1978; Baxter *et al.*, 1979). However, other studies

Abbreviations used: PDH_a , the active, non-phosphorylated form of the pyruvate dehydrogenase complex; $k_{0.5}$, concentration of effector required for half-maximal response.

suggest that the major reason for the loss of response to pyruvate in these conditions is an increase in the overall activity of pyruvate dehydrogenase kinase (Hutson & Randle, 1978). More recently, evidence for the presence of increased amounts of a novel activating factor for pyruvate dehydrogenase kinase in mitochondria from the hearts of starved and alloxan-diabetic rats has been presented (Kerbey & Randle, 1981).

Increases in the concentration of Ca^{2+} in the medium have also been shown to lead to increases in the amount of PDH_a both in intact heart mitochondria and in perfused heart preparations from normal fed rats (Denton *et al.*, 1980; McCormack & Denton, 1981a). Adrenaline has been shown to increase the amount of PDH_a in perfused hearts from normal fed rats (Hiraoka *et al.*, 1980; McCormack & Denton, 1981a). The most likely explanation for this effect of adrenaline is that the hormone increases the cytoplasmic concentration of Ca^{2+} , which would then lead to increases in the amount of PDH_a by activation of pyruvate dehydrogenase phosphate phosphatase (Hiraoka *et al.*, 1980; McCormack & Denton, 1981a). McCormack & Denton (1981a) also noted that the increases in the amount of PDH_a caused by adrenaline were much smaller in hearts from starved or alloxan-diabetic rats, unless pyruvate was present in the perfusion medium. These observations suggested that the rat heart pyruvate dehydrogenase system may become less responsive to Ca^{2+} in starvation and alloxan-diabetes and that there may be complex interactions between the effects of pyruvate and Ca^{2+} in the regulation of the amount of PDH_a .

The oxoglutarate dehydrogenase complex of rat heart and other mammalian mitochondria is activated by Ca^{2+} in the same concentration range as pyruvate dehydrogenase phosphate phosphatase (McCormack & Denton, 1979, 1980, 1981b; Denton & McCormack, 1980). The effect of Ca^{2+} on this enzyme is direct and does not involve regulation by reversible phosphorylation (McCormack & Denton, 1979). We have therefore utilized the Ca^{2+} -sensitive properties of the oxoglutarate dehydrogenase complex to estimate the intramitochondrial concentration of Ca^{2+} and thereby to assess the concentration gradient of Ca^{2+} ions across the mitochondrial inner membrane under the various conditions of the present studies.

Experimental

Normal rats (male, Wistar, 250–300 g) were fed *ad libitum* on a stock laboratory diet (Breeding Diet; Oxoid, Basingstoke, Hants., U.K.). Starved rats were deprived of food for 48 h. Diabetes was induced by intravenous injection of alloxan (60 mg/kg) under light ether anaesthesia 48 h before use.

Hearts were perfused at 37°C as described by

McCormack & Denton (1981a) and mitochondria were prepared as in Denton *et al.* (1980). Mitochondria were incubated at 30°C (0.5–1 mg of protein/ml of incubation medium) in 125 mM-KCl/20 mM-NaCl/20 mM-Tris/HCl (pH 7.3)/5 mM- KH_2PO_4 with further additions as indicated; in some experiments NaCl was absent (see the text, and Figure and Table legends). All incubations were in 1 ml, except those with the double-beam spectrophotometer, which were in 2 ml (see below).

Pyruvate dehydrogenase activity was assayed with arylamine acetyltransferase as described by Coore *et al.* (1971) in extracts of tissue or mitochondria, which were prepared as described by McCormack & Denton (1981a and 1980 respectively). Total pyruvate dehydrogenase activity was determined after treatment of extracts with pyruvate dehydrogenase phosphate phosphatase (McCormack & Denton, 1980). The total activity was not affected significantly by any of the conditions tested and ranged from 2 to 4 units/g wet wt. of tissue and from 80 to 120 munits/mg of mitochondrial protein. A unit is defined as the amount of enzyme catalysing the conversion of pyruvate into acetyl-CoA at the rate of 1 $\mu\text{mol/min}$ at 30°C.

Concentrations of Ca^{2+} were generated by using EGTA buffers and free Ca^{2+} concentrations were calculated as described by Denton *et al.* (1978) and McCormack & Denton (1979); it should be noted that these may be underestimated by a factor of 1.8 (see Tsien & Rink, 1980). Great care was taken to ensure that the pH of EGTA-containing medium was not altered by the various additions, as this would markedly alter the free Ca^{2+} concentrations (see Denton *et al.*, 1978).

Oxygen uptake was measured with a Clark-type oxygen electrode as described by McCormack & Denton (1980). Mitochondrial protein was measured by the method of Gornall *et al.* (1949) and mitochondrial ATP content by the method of Stanley & Williams (1969) as described by McCormack & Denton (1980).

The extent of mitochondrial NAD(P) reduction was followed on a continuous-recording double-beam spectrophotometer (by kind permission of Professor J. B. Chappell of this Department) at a wavelength pair of 355 and 370 nm.

Alloxan was from Kodak, Kirkby, Liverpool, U.K. All other chemicals and biochemicals were obtained from either Boehringer, Lewes, East Sussex, U.K., or BDH Chemicals, Poole, Dorset, U.K.

Results and discussion

Effects of starvation and diabetes on the response of PDH_a in the perfused rat heart to adrenaline, Ca^{2+} and pyruvate

The addition of either adrenaline or pyruvate to the perfusion medium, or increasing the Ca^{2+} in the

medium from 1.2 to 6 mM, results in 3–4-fold increases in the amount of PDH_a in hearts from normal fed rats, whereas much diminished responses of PDH_a to these effectors are observed in perfused hearts from starved or alloxan-diabetic rats (McCormack & Denton, 1981a; Table 1). However, McCormack & Denton (1981a) observed that the addition of pyruvate to the perfusion medium allowed the PDH_a in hearts from starved and diabetic rats to respond to adrenaline in a manner similar to that exhibited in hearts from normal rats. The results given in Table 1 confirm and extend this finding of apparent reciprocal amplification exhibited by pyruvate and adrenaline or higher Ca²⁺ in the medium in their effects on the amount of heart PDH_a.

Effects of starvation and diabetes on the responses of PDH_a in isolated rat heart mitochondria to Ca²⁺, pyruvate and ADP

Previous studies with coupled heart mitochondria from normal fed rats incubated with an oxidizable substrate demonstrated that increases in extramitochondrial Ca²⁺ concentration could lead to increases in the steady-state amounts of PDH_a [from about 10 to 50% of total pyruvate dehydrogenase activity (Denton *et al.*, 1980; Hansford, 1981)]. This is probably achieved by Ca²⁺ activation of pyruvate dehydrogenase phosphate phosphatase (Denton *et al.*, 1972), though inhibition of pyruvate dehydrogenase kinase by Ca²⁺ may also be involved (Cooper *et al.*, 1974). The range of extramitochondrial Ca²⁺ concentrations (generated by using CaEGTA buffers) to which the pyruvate dehydrogenase system responded was found to be dependent on the presence or absence of effectors of the Ca²⁺-transport system of the mitochondrial inner

membrane, in particular Na⁺ and Mg²⁺ (see Carafoli, 1979; Denton & McCormack, 1980). In the studies reported in the present paper, a saturating amount of Na⁺ (20 mM) has often been added to the incubations, so that the addition of pyruvate as a sodium salt had no effect on the concentration of Ca²⁺ to which the enzyme is exposed inside the mitochondria.

In a preliminary series of time-course experiments (results not shown) it was found that increases in the extramitochondrial concentrations of either Ca²⁺ or pyruvate resulted in new, increased, steady-state amounts of PDH_a within 2 min. The activation initiated by Ca²⁺ was appreciably faster than that by pyruvate. This is to be expected, as Ca²⁺ (by activating the phosphatase) will increase the turnover of the pyruvate dehydrogenase phosphorylation–dephosphorylation cycle, whereas pyruvate (by inhibiting the kinase) will decrease this. The time-course for the decrease in the amount of PDH_a resulting from a lowering of the extramitochondrial concentration of Ca²⁺ (in the presence of Na⁺) was found to be essentially complete after 4 min (results not shown). This demonstrated that the effects of Ca²⁺ are fully reversible under these conditions (see also Hansford, 1981). However, if Na⁺ were absent, reversal occurred very slowly and was not complete for more than 10 min (results not shown). These observations on the reversibility of the Ca²⁺ effect on the pyruvate dehydrogenase system are similar to those obtained (in the absence and presence of Na⁺) for the Ca²⁺ effect on oxoglutarate oxidation by coupled rat heart mitochondria (Denton *et al.*, 1980). In subsequent experiments, mitochondria were incubated for both 4 and 8 min to ensure that steady-state values of PDH_a were achieved. Since essentially similar results were

Table 1. *Effects of pyruvate, adrenaline and high Ca²⁺ concentration in medium on the amount of PDH_a in perfused hearts of normal, starved or diabetic rats*

Hearts were perfused at 7 kPa for 10 min with bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 10 mM-glucose with or without 5 mM-pyruvate as indicated. Perfusions were then continued for 1 min with additions of 1 μ M-adrenaline or higher Ca²⁺ in the medium (from 1.2 to 6 mM) as indicated. In the latter case only 0.2 mM-KH₂PO₄ (rather than 1.2 mM) was present in the perfusion medium to avoid calcium phosphate precipitation; this lowering of [KH₂PO₄] had no effect on any of the other conditions (results not shown). Results are given as means $\pm$ S.E.M. for the numbers of perfusions in parentheses. * $P \leq 0.01$ and ** $P \leq 0.001$ for the effect of the additions compared with the appropriate control value by Student's *t* test. In every condition the values obtained for the starved or diabetic conditions were significantly less ($P \leq 0.001$) than the corresponding value for the normal condition.

Additions	PDH _a (as % of total activity) in hearts from:		
	Normal rats	Starved rats	Diabetic rats
None (control)	9.2 $\pm$ 0.9 (6)	2.5 $\pm$ 0.9 (5)	2.0 $\pm$ 0.6 (4)
5 μ M-adrenaline	32.3 $\pm$ 2.1 (4)**	3.7 $\pm$ 1.9 (5)	6.7 $\pm$ 1.8 (4)
5 mM-pyruvate	35.2 $\pm$ 2.1 (5)**	5.8 $\pm$ 1.1 (4)	4.9 $\pm$ 1.2 (4)
Ca ²⁺ in medium raised to 6 mM	33.1 $\pm$ 3.4 (5)*	3.0 $\pm$ 0.8 (3)	3.9 $\pm$ 0.6 (3)
5 μ M-adrenaline plus 5 mM-pyruvate	96.0 $\pm$ 2.4 (4)**	37.8 $\pm$ 3.3 (4)*	37.0 $\pm$ 2.1 (4)**
Ca ²⁺ in medium raised to 6 mM, plus 5 mM-pyruvate	95.0 $\pm$ 2.0 (3)**	30.3 $\pm$ 2.9 (3)**	33.2 $\pm$ 3.1 (3)**

obtained at both times, only results after 4 min incubation are given. Hutson *et al.* (1978) have previously reported that the sensitivity to pyruvate of the pyruvate dehydrogenase system in heart mitochondria from normal fed rats was greatly diminished after preincubation in the presence of EGTA for 6 min. We have found no evidence for this phenomenon in preincubations up to 5 min (results not shown). We have no explanation for this discrepancy, as the present study and that of Hutson *et al.* (1978) have been performed under similar conditions.

The effects of Ca^{2+} on the response of PDH_a to pyruvate, and vice versa, in intact heart mitochondria from normal and starved rats are shown in Fig. 1. In the presence of EGTA ($\text{Ca}^{2+} < 1 \text{ nM}$) the extent of the increase in the amount of PDH_a caused by pyruvate was much diminished in the mitochondria from starved rats (Fig. 1a; cf. symbols $\circ$ and $\bullet$); this has been shown previously (Kerbey *et al.*, 1976, 1977; Hutson *et al.*, 1978). Fig. 1(b) shows that, in the absence of pyruvate (cf. symbols $\circ$ and $\bullet$), Ca^{2+} caused only a modest increase in the amount of PDH_a in mitochondria from starved rats compared with that exhibited in mitochondria from control rats. However, the response of PDH_a to Ca^{2+} in mitochondria from starved rats can be greatly enhanced by the inclusion of increasing

concentrations of pyruvate in the incubation medium. For example, the presence of 0.25 mM-pyruvate in the medium of mitochondria from starved rats (Fig. 1b, $\blacksquare$) resulted in a response of PDH_a to changes in the extramitochondrial concentrations of Ca^{2+} which was very similar to that observed in mitochondria from normal rats incubated in the absence of pyruvate (Fig. 1b, $\circ$). This effect of pyruvate on the response of PDH_a to Ca^{2+} did not appear to involve any major changes in the $k_{0.5}$ values for Ca^{2+} , but rather appeared to alter the magnitude of the overall response of PDH_a to changes in extramitochondrial Ca^{2+} concentrations. All the calculated $k_{0.5}$ values for Ca^{2+} for the traces shown in Fig. 1(b) were in the range 100–200 nM. Similarly, the inclusion of a low concentration of Ca^{2+} in the incubation medium (e.g. 120 nM; Fig. 1a, $\blacksquare$) resulted in the response of PDH_a to pyruvate in mitochondria from starved rats becoming similar to that observed in mitochondria from normal rats incubated at less than 1 nM- Ca^{2+} (Fig. 1a, $\circ$).

Further examples of the interactions of Ca^{2+} and pyruvate in the regulation of pyruvate dehydrogenase activity within intact rat heart mitochondria are given in Table 2. The results of Table 2 show that PDH_a in incubated mitochondria from alloxan-diabetic rats responded to additions of Ca^{2+} and/or pyruvate to the medium in a manner which was

Fig. 1. Effects of Ca^{2+} concentration in the absence and presence of pyruvate, and vice versa, on the amount of PDH_a in heart mitochondria from normal and starved rats

Mitochondria were incubated (for 4 min) as described in the Experimental section in KCl-based medium containing 10 mM-oxoglutarate, 0.25 mM-malate and 5 mM-EGTA and in the presence of the concentrations of pyruvate or Ca^{2+} (generated by using 5 mM-EGTA buffers) indicated on the abscissae of (a) and (b) respectively. In both (a) and (b) the open symbols represent the normal condition and the closed symbols the starved condition. Further additions were as follows: in (a) none ($\bullet$, $\circ$; $[\text{Ca}^{2+}] < 1 \text{ nM}$); 2.5 mM- CaCl_2 ($\blacktriangle$, $\triangle$; $[\text{Ca}^{2+}] \approx 50 \text{ nM}$); 3.5 mM- CaCl_2 ($\blacksquare$, $\square$; $[\text{Ca}^{2+}] \approx 120 \text{ nM}$); 4 mM- CaCl_2 ($\blacktriangledown$, $\triangledown$; $[\text{Ca}^{2+}] \approx 210 \text{ nM}$); and in (b) none ($\bullet$, $\circ$); 0.1 mM-pyruvate ($\blacktriangle$, $\triangle$); 0.25 mM-pyruvate ($\blacksquare$, $\square$); and 0.5 mM-pyruvate ($\blacktriangledown$, $\triangledown$). Each point shown represents the mean of observations made on at least three different preparations of mitochondria.

Table 2. Interactions of Ca²⁺ and pyruvate in their effects on PDH_a in heart mitochondria from normal, starved and diabetic rats incubated under a variety of conditions. Mitochondria were incubated (for 4 min) as described in the Experimental section in KCl-based medium containing additions as indicated. Results are given as means $\pm$ S.E.M. for the numbers of separate observations (each made on a different preparation of mitochondria) given in parentheses.

Other additions		PDH _a (as % of total activity) in mitochondria from:					
		Normal rats incubated with		Starved rats incubated with		Diabetic rats incubated with	
	[Pyruvate] (mM)	5 mM-EGTA ([Ca ²⁺] < 1 nM)	5 mM-EGTA plus 4 mM-CaCl ₂ ([Ca ²⁺] ≈ 210 mM)	5 mM-EGTA ([Ca ²⁺] < 1 nM)	5 mM-EGTA plus 4 mM-CaCl ₂ ([Ca ²⁺] ≈ 210 nM)	5 mM-EGTA ([Ca ²⁺] < 1 nM)	5 mM-EGTA plus 4 mM-CaCl ₂ ([Ca ²⁺] ≈ 210 nM)
10 mM-oxoglutarate plus 0.25 mM-malate	0	10.2 ± 1.3 (6)	44.2 ± 3.5 (5)	0.5 ± 0.2 (5)	8.4 ± 0.6 (4)	<0.2 (3)	6.6 ± 0.7 (3)
	0.1	16.0 ± 1.8 (3)	72.2 ± 2.5 (3)	0.7 ± 0.2 (3)	20.6 ± 1.5 (3)	<0.2 (3)	13.8 ± 0.7 (3)
	0.5	40.5 ± 1.4 (3)	102.5 ± 2.8 (3)	3.2 ± 0.7 (3)	63.0 ± 3.5 (3)	0.8 ± 0.1 (3)	31.7 ± 1.9 (3)
10 mM-glutamate plus 0.5 mM-malate	0	13.5 ± 1.5 (4)	48.6 ± 2.1 (4)	4.1 ± 0.8 (3)	17.2 ± 0.6 (3)	3.2 ± 0.2 (3)	9.8 ± 0.5 (3)
	0.25	29.1 ± 3.2 (3)	103.1 ± 4.7 (3)	8.8 ± 1.3 (3)	62.4 ± 2.9 (3)	3.6 ± 0.7 (3)	34.4 ± 3.7 (3)
	0	7.6 ± 0.7 (3)	54.4 ± 2.7 (5)	0.3 ± 0.1 (3)	21.0 ± 1.2 (3)	<0.2 (3)	8.8 ± 0.9 (3)
10 mM-succinate	0.25	35.0 ± 3.0 (3)	96.5 ± 1.6 (3)	0.5 ± 0.1 (3)	59.8 ± 3.1 (3)	0.4 ± 0.2 (3)	39.8 ± 3.2 (3)
10 mM-succinate plus 1 mM-MgCl ₂	0	12.1 ± 1.9 (3)	26.7 ± 2.9 (3)	0.4 ± 0.2 (3)	1.6 ± 0.4 (3)	<0.3 (3)	1.2 ± 0.4 (3)
	0.25	36.2 ± 2.6 (3)	80.0 ± 4.1 (3)	0.5 ± 0.3 (3)	23.7 ± 7.3 (3)	<0.2 (3)	14.8 ± 1.3 (3)

similar to that found for PDH_a in mitochondria from starved rats. The responses of PDH_a in the three types of mitochondria were essentially unaffected by the replacement of oxoglutarate as the main oxidizable substrate by either succinate or glutamate plus malate (Table 2). The presence of Mg²⁺ (see Denton *et al.*, 1980) did not appear to affect the ability of pyruvate to enhance the response of PDH_a to Ca²⁺ (and vice versa) in the three types of mitochondria (Table 2).

The pyruvate analogue dichloroacetate (Whitehouse *et al.*, 1974) gave similar results to pyruvate in both coupled mitochondria (as under the conditions shown in Table 2) or in uncoupled mitochondria (where pyruvate would be rapidly oxidized) incubated with 1 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 5 mM-MgATP and 10 μ g of oligomycin/ml (results not shown). These experiments suggested that the metabolism of pyruvate was not necessary to observe the effects described above, and also that changes in the components of the mitochondrial protonmotive force were unlikely to be involved.

The results given in Table 3 illustrate that the activation of PDH_a on addition of ADP was also diminished in heart mitochondria from starved rats. However, the response was greatly enhanced in these mitochondria by the presence of low concentrations of Ca²⁺ and/or pyruvate in the incubation medium. In the presence of 0.1 mM-pyruvate and 120 nM-Ca²⁺, the increase in PDH_a on addition of 0.1 mM-ADP to mitochondria from starved rats was even greater than that observed in control mitochondria incubated in the absence of added pyruvate and Ca²⁺. Similar results were obtained with mitochondria prepared from the hearts of diabetic rats (results not shown). It has been suggested previously, on the basis of studies with intact isolated liver cells, that starvation and diabetes may result in the response of the pyruvate dehydrogenase system to ADP becoming decreased (Siess & Wieland, 1976).

Kerbey *et al.* (1977) reported that heart mitochondria prepared from starved or diabetic rats had lower ATP content and coupling ratios (as defined by Chance & Williams, 1956) than did those prepared from normal rats. In the present studies we could find no significant differences. Mean values for ATP content were similar to those found by Denton *et al.* (1980), ranging between 7.2 and 8.5 nmol/mg of protein, and were apparently unaltered by starvation or diabetes or by the addition of pyruvate up to 2 mM or Ca²⁺ up to 210 nM. Coupling ratios determined with oxoglutarate plus malate as substrate were 5.9 $\pm$ 0.7, 6.4 $\pm$ 0.7 and 5.4 $\pm$ 0.7 (means $\pm$ S.E.M. for six to eight observations) for mitochondria from normal, starved and diabetic rats respectively.

Table 3. *Effects of ADP in the absence and presence of pyruvate and/or Ca²⁺ on the amount of PDH_a in heart mitochondria from normal and starved rats*

Mitochondria were incubated (for 4 min) as described in the Experimental section in KCl-based medium containing 10 mM-oxoglutarate, 0.25 mM-malate, 5 mM-EGTA and 5 µg of oligomycin/ml. Other additions were as indicated. Results are given as means ± S.E.M. for three observations, each made on a different preparation of mitochondria.

Additions	PDH _a (as % of total activity) in mitochondria from:	
	Normal rats	Starved rats
None	12.6 ± 2.0	0.5 ± 0.3
0.1 mM-ADP	21.5 ± 3.2	0.4 ± 0.3
0.25 mM-ADP	46.8 ± 5.4	1.4 ± 0.4
2 mM-ADP	68.7 ± 4.0	6.7 ± 1.2
3.5 mM-CaCl ₂ ([Ca ²⁺] ≈ 120 nM)	29.8 ± 3.4	1.7 ± 0.6
0.1 mM-pyruvate	17.8 ± 1.7	0.4 ± 0.2
0.1 mM-ADP plus 3.5 mM-CaCl ₂	53.2 ± 4.3	15.5 ± 1.0
0.1 mM-ADP plus 0.1 mM-pyruvate	51.3 ± 5.6	6.5 ± 1.2
0.1 mM-pyruvate plus 3.5 mM-CaCl ₂	66.6 ± 4.3	5.9 ± 1.2
0.1 mM-ADP plus 0.1 mM-pyruvate plus 3.5 mM-CaCl ₂ ([Ca ²⁺] ≈ 120 nM)	94.9 ± 5.1	46.4 ± 4.4

Use of the activity of the oxoglutarate dehydrogenase complex to estimate possible changes in intramitochondrial Ca²⁺ concentrations

Earlier studies with intact coupled mitochondria from rat heart have shown that changes in the oxidation of oxoglutarate can be used as a means of estimating the activity of the oxoglutarate dehydrogenase complex within the mitochondria, and that changes in the intramitochondrial concentration of Ca²⁺ can alter the K_m for the oxidation of oxoglutarate (Denton *et al.*, 1980).

In the presence of ADP, the maximal rate of oxoglutarate oxidation (in the presence or absence of Ca²⁺) at 20 mM-oxoglutarate is unaffected by starvation or diabetes (see the legend to Table 4). This is in contrast with the oxidation of pyruvate, which is much depressed by starvation or diabetes (results not shown). In these experiments mitochondria were incubated in KCl-based media with 1 mM-malate, 5 mM-EGTA, 1 µM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 5 mM-MgATP (to allow kinase activity) and 5 µg of oligomycin/ml, and the initial rate of oxidation of 2 mM-pyruvate (as nmol of O₂ taken up in the first min per mg of protein) was determined after a 1 min preincubation. The results were, in mitochondria from normal rats, 164 ± 16, from starved rats 21 ± 5*, from diabetic rats, 36 ± 6* (as means ± S.E.M. for three observations on different preparations of mitochondria; * P ≤ 0.01 for the effects of starvation or diabetes, by Student's *t* test).

At a low concentration of oxoglutarate (0.5 mM) the effects of Ca²⁺, Na⁺ and Mg²⁺ on oxidation in the presence of ADP do not appear to be affected by starvation or diabetes (Table 4a). Therefore it is unlikely that the distribution of Ca²⁺ across the

mitochondrial inner membrane, or the properties of the Ca²⁺-transport system in this membrane, are affected by starvation or diabetes under these conditions. However, these studies (in Table 4a) required the presence of ADP, whereas in most of the studies where the amounts of PDH_a in mitochondria were determined no ADP was present (Fig. 1; Table 2). We therefore determined the extent of oxoglutarate-induced changes in the NAD(P)H content of mitochondria as a convenient means of monitoring oxoglutarate dehydrogenase activity in mitochondria incubated in the absence of ADP. These determinations were made with 1 mM-oxoglutarate (in the presence of malate), which, under the conditions used, always gave increases in NAD(P)H content that were below the maximal NAD(P)H content that could be achieved (see legend to Fig. 2). Shortly after the submission of the present paper, a report by Hansford & Castro (1981) was published in which those authors also followed the reduction of NAD(P) to monitor the effects of Ca²⁺ on oxoglutarate dehydrogenase activity in intact rat heart mitochondria.

Fig. 2 shows that increases in the extramitochondrial concentration of Ca²⁺ lead to increases in the content of NAD(P)H, resulting from the addition of oxoglutarate to incubated heart mitochondria from normal fed rats. Starvation or diabetes had little effect on these responses under various incubation conditions (Table 4b).

It was not feasible to assess the effect that pyruvate may have on the distribution of Ca²⁺ ions across the mitochondrial inner membrane by using the activity of the oxoglutarate dehydrogenase complex within mitochondria. However, dichloro-

Fig. 2. Oxoglutarate-induced reduction of mitochondrial NAD(P) in the presence of various concentrations of Ca²⁺

Heart mitochondria from normal rats were incubated as described in the Experimental section in KCl-based medium containing 5 mM-EGTA and additions of CaCl₂ to yield the following Ca²⁺ concentrations: 1, <1 nM; 2, 50 nM; 3, 120 nM; 4, 210 nM; 5, 470 nM (the numbers refer to the traces as indicated). The mitochondria were preincubated for 3 min (as shown), by which time no further oxidation of endogenous substrate was taking place, and then 0.25 mM-malate and 1 mM-oxoglutarate were added. Results from a typical experiment are shown. The oxoglutarate itself absorbed at the wavelength pair used; however, this has been omitted for clarity of presentation and only NAD(P) reduction is shown. A 100% reduction refers to that achieved with 2 mM-oxoglutarate in the presence of 700 nM-Ca²⁺; further addition of oxoglutarate did not increase this under this condition. A similar amount of reduction was observed with either 10 mM-glutamate plus 2 mM-malate or 20 μ M-palmitoylcarnitine plus 1 mM-malate.

Na⁺ were, for the normal, starved and diabetic conditions respectively: in the presence of ADP, 137 ± 14 , 117 ± 11 and 130 ± 8 ; in the absence of ADP, 141 ± 8 , 133 ± 9 and 121 ± 7 (results are given as mean $k_{0.5}$ values $\pm$ S.D. for at least 20 observations made at six to eight different Ca²⁺ concentrations on mitochondria from three different preparations in each case). An important additional inference that can be drawn from these observations is that the distribution of Ca²⁺ across the mitochondrial inner membrane is unlikely to be markedly different in the presence or absence of ADP.

General discussion

Earlier studies have demonstrated the diminution in response of the pyruvate dehydrogenase system to activation by pyruvate in starvation or diabetes, by using both perfused hearts and isolated heart mitochondria (Kerbey *et al.*, 1976, 1977; Hutson & Randle, 1978; Hutson *et al.*, 1978). The present study has shown, with both preparations, that starvation or diabetes also results in a much diminished response of the pyruvate dehydrogenase system to Ca²⁺ (Fig. 1, Tables 1 and 2). In addition, evidence for a diminished response to ADP has been obtained with isolated mitochondria (Table 3).

The diminished responses to effectors in starvation or diabetes appear to reside within the pyruvate dehydrogenase system itself rather than in any more general change in mitochondrial function, which, for example, might alter the distribution of Ca²⁺ or other effectors across the mitochondrial inner membrane. Not only were no appreciable alterations in mitochondrial respiratory-control ratios or in intramitochondrial ATP contents detected, but no differences were apparent in the activity of the oxoglutarate dehydrogenase complex within heart mitochondria from control, starved or diabetic rats. This last approach seems especially powerful in the present context, since any alteration in the distribution of either Ca²⁺ or H⁺ across the mitochondrial inner membrane would have resulted in changes in the activity of oxoglutarate dehydrogenase at low extramitochondrial concentrations of oxoglutarate (Denton *et al.*, 1980; Halestrap, 1978; Palmieri *et al.*, 1972). Since no such changes were observed, it seems reasonable to conclude that changes in the distribution of these cations do not contribute to the loss of response to Ca²⁺ and pyruvate.

The results of the present study also show that the loss of responses to Ca²⁺ and pyruvate of the pyruvate dehydrogenase system in starvation or diabetes is not due to any diminution in the intrinsic sensitivities of the phosphatase or kinase to Ca²⁺ or pyruvate respectively. Restoration of the response to either Ca²⁺ or pyruvate has been found in isolated mitochondria in the presence of a low concentration

acetate (up to 2 mM) did not appreciably alter the effects of Ca²⁺ on oxoglutarate oxidation or on oxoglutarate-induced changes in NAD(P)H content under the conditions shown in Table 4.

In both the presence and absence of ADP (Table 4) the responses of the intramitochondrial oxoglutarate dehydrogenase complex to changes in the extramitochondrial concentrations of Ca²⁺, Na⁺ and Mg²⁺ were similar. For example, calculated $k_{0.5}$ values (nM) for the effects of Ca²⁺ in the presence of

Table 4. *Effects of starvation and diabetes on (a) the initial rate of oxoglutarate oxidation by, and (b) the extent of reduction of NAD(P) in, rat heart mitochondria incubated in the presence and absence of Na⁺, Mg²⁺ and Ca²⁺*

Mitochondria were incubated as described in the Experimental section in KCl-based medium containing 5 mM-EGTA and additions of CaCl₂ to give the Ca²⁺ concentrations shown and with additions of Na⁺ and Mg²⁺ as indicated; 1 mM-malate and 2 mM-ADP were also present in (a). Mitochondria were preincubated for either (a) 1 min or (b) 3 min and then either (a) 0.5 mM-oxoglutarate or (b) 1 mM-oxoglutarate plus 1 mM-malate were added. Values were derived as follows: (a) rates of oxidation in the absence of substrate (16–24 nmol of O₂ taken up/min per mg of protein) were subtracted to give substrate-dependent rates; initial rates of oxidation of 0.5 mM-oxoglutarate are expressed as percentages of the initial rates of oxidation of a saturating amount (20 mM) of oxoglutarate; these maximal rates were 80 ± 4 (7), 75 ± 6 (7) and 83 ± 7 (5) nmol of O₂ taken up/min per mg of protein for the normal, starved and diabetic conditions respectively (as means ± S.E.M. for the numbers of observations made on different preparations of mitochondria in parentheses); (b) measurements of the extent of NAD(P) reduction were as given in the legend to Fig. 2, 2 min after the addition of substrate. Results are given as means ± S.E.M. for either (a) four or (b) three observations on different preparations of mitochondria.

Additions			(a) Rate of oxidation of 0.5 mM-oxoglutarate (as % of maximum rate of oxoglutarate oxidation) in mitochondria from:			(b) Extent of reduction of NAD(P) (as % of maximum reduction) in mitochondria from:		
[Na ⁺] (mM)	[Mg ²⁺] (mM)	[Ca ²⁺] (nM)	Normal rats	Starved rats	Diabetic rats	Normal rats	Starved rats	Diabetic rats
0	0	<1	20 ± 2	20 ± 2	20 ± 2	18 ± 3	18 ± 2	18 ± 3
0	0	50	55 ± 6	57 ± 6	56 ± 4	83 ± 8	77 ± 2	79 ± 5
20	0	<1	20 ± 3	21 ± 3	20 ± 2	20 ± 3	18 ± 2	17 ± 2
20	0	50	28 ± 3	28 ± 3	26 ± 3	29 ± 4	31 ± 4	29 ± 5
20	0	210	55 ± 6	59 ± 6	57 ± 7	76 ± 8	77 ± 4	77 ± 7
0	1	<1	23 ± 3	22 ± 3	21 ± 2	20 ± 3	19 ± 4	18 ± 2
0	1	50	31 ± 3	30 ± 3	32 ± 2	27 ± 5	25 ± 4	25 ± 3
20	1	<1	22 ± 2	23 ± 2	22 ± 2	23 ± 1	20 ± 4	21 ± 2
20	1	210	32 ± 4	36 ± 2	32 ± 4	33 ± 4	30 ± 3	32 ± 5

of the other effector (Fig. 1, Table 2). This pattern of reciprocal amplification matched closely the pattern found in the perfused heart. Our results are compatible with there being an increase in the maximum activity of the kinase, relative to that of the phosphatase, in starvation or diabetes. Kerbey & Randle (1981) have reported evidence that, in both starvation and diabetes, there is an increase in the amount (activity) of a non-dialysable thermolabile activator of pyruvate dehydrogenase kinase within heart mitochondria. Another factor that may be important is the intramitochondrial content of K⁺, since Roche & Reed (1974), observed that the ADP inhibition of pyruvate dehydrogenase kinase, in a purified system, could be enhanced on increasing the concentration of K⁺ in the range 30–90 mM. Whatever the mechanism, it seems likely that it may be a long-term consequence of diabetes or starvation and quite distinct from that whereby insulin brings about the short-term activation of pyruvate dehydrogenase in fat-cells (Denton *et al.*, 1971; Jungas, 1971; Weiss *et al.*, 1971). This effect of insulin is not fully understood, but involves activation of the phosphatase rather than a primary change in kinase activity (Hughes & Denton, 1976).

Finally, the results of the present study give further support for the view that adrenaline increases

the amount of PDH_a in the rat heart via an increase in the intramitochondrial concentration of Ca²⁺. Increasing the concentration of Ca²⁺ in the perfusion medium has been shown to mimic closely the effect of adrenaline on the amount of PDH_a not only in hearts from normal fed animals, but also from starved and diabetic animals. The responses to both adrenaline and increased Ca²⁺ in the medium are diminished by starvation or diabetes unless pyruvate is present in the perfusion medium, a pattern of response also evident in isolated mitochondria.

The α-subunits of purified pyruvate dehydrogenase complex contain three phosphorylation sites, only one of which appears to be responsible for the decreases in activity caused by phosphorylation (see Sugden *et al.*, 1979; Yeaman *et al.*, 1978). Evidence for the phosphorylation of all three sites has been obtained for both isolated mitochondria and intact cell preparations (Hughes & Denton, 1978; Hughes *et al.*, 1980; Sale & Randle, 1980, 1981). Measurements of the site occupancy under the various conditions of the present study should throw light on the role of the other two phosphorylation sites in the regulation of the pyruvate dehydrogenase system.

We are grateful to Dr. Paul England (this Department) for making available computer programs, Professor J. B.

Chappell (this Department) for allowing us to use his double-beam spectrophotometer, Mr. Philip Drake for the care of animals and the Medical Research Council for financial support.

References

- Baxter, M. A. & Coore, H. G. (1978) *Biochem. J.* **174**, 553–561
- Baxter, M. A., Goheer, M. A. & Coore, H. G. (1979) *FEBS Lett.* **97**, 27–31
- Carafoli, E. (1979) *FEBS Lett.* **104**, 1–5
- Chance, B. & Williams, G. R. (1956) *Adv. Enzymol.* **17**, 65–134
- Cooper, R. H., Randle, P. J. & Denton, R. M. (1974) *Biochem. J.* **143**, 625–641
- Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* **125**, 115–127
- Denton, R. M. (1975) *Proc. Nutr. Soc.* **34**, 217–224
- Denton, R. M. & Hughes, W. A. (1978) *Int. J. Biochem.* **9**, 545–552
- Denton, R. M. & McCormack, J. G. (1980) *FEBS Lett.* **119**, 1–8
- Denton, R. M., Coore, H. G., Martin, B. R. & Randle, P. J. (1971) *Nature (London)* **231**, 115–116
- Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163
- Denton, R. M., Randle, P. J., Bridges, B. J., Cooper, R. H., Kerbey, A. L., Pask, H. T., Severson, D. L., Stansbie, D. & Whitehouse, S. (1975) *Mol. Cell. Biochem.* **9**, 27–53
- Denton, R. M., Richards, D. A. & Chin, J. G. (1978) *Biochem. J.* **176**, 899–906
- Denton, R. M., McCormack, J. G. & Edgell, N. J. (1980) *Biochem. J.* **190**, 107–117
- Gornall, H. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–756
- Halestrap, A. P. (1978) *Biochem. J.* **172**, 377–387
- Hansford, R. G. (1981) *Biochem. J.* **194**, 721–732
- Hansford, R. G. & Castro, F. (1981) *Biochem. J.* **198**, 525–533
- Hiraoka, T., De Buysere, M. & Olson, M. S. (1980) *J. Biol. Chem.* **255**, 7604–7609
- Hughes, W. A. & Denton, R. M. (1976) *Nature (London)* **264**, 471–473
- Hughes, W. A. & Denton, R. M. (1978) *Biochem. Soc. Trans.* **6**, 1228–1230
- Hughes, W. A., Brownsey, R. W. & Denton, R. M. (1980) *Biochem. J.* **192**, 469–481
- Hutson, N. J. & Randle, P. J. (1978) *FEBS Lett.* **92**, 73–76
- Hutson, N. J., Kerbey, A. L., Randle, P. J. & Sugden, P. H. (1978) *Biochem. J.* **173**, 669–680
- Jungas, R. L. (1971) *Metab. Clin. Exp.* **20**, 43–53
- Kerbey, A. L. & Randle, P. J. (1981) *FEBS Lett.* **127**, 188–192
- Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S. Pask, H. T. & Denton, R. M. (1976) *Biochem. J.* **154**, 327–348
- Kerbey, A. L., Radcliffe, P. M. & Randle, P. J. (1977) *Biochem. J.* **164**, 509–519
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- McCormack, J. G. & Denton, R. M. (1979) *Biochem. J.* **180**, 533–544
- McCormack, J. G. & Denton, R. M. (1980) *Biochem. J.* **190**, 95–105
- McCormack, J. G. & Denton, R. M. (1981a) *Biochem. J.* **194**, 639–643
- McCormack, J. G. & Denton, R. M. (1981b) *Biochem. J.* **196**, 619–624
- Palmieri, F., Quagliariello, E. & Klingenberg, M. (1972) *Eur. J. Biochem.* **29**, 408–416
- Randle, P. J., Hutson, N. J., Kerbey, A. L. & Sugden, P. H. (1979) *Miami Winter Symp.* **16**, 501–520
- Roche, T. E. & Reed, L. J. (1974) *Biochem. Biophys. Res. Commun.* **59**, 1341–1348
- Sale, G. J. & Randle, P. J. (1980) *Biochem. J.* **188**, 409–421
- Sale, G. J. & Randle, P. J. (1981) *Biochem. J.* **193**, 935–946
- Siess, E. A. & Wieland, O. H. (1976) *Biochem. J.* **156**, 91–102
- Stanley, P. E. & Williams, S. G. (1969) *Anal. Biochem.* **29**, 381–392
- Sugden, P. H., Kerbey, A. L., Randle, P. J., Waller, C. A. & Reed, K. B. M. (1979) *Biochem. J.* **181**, 419–426
- Tsien, R. Y. & Rink, T. J. (1980) *Biochim. Biophys. Acta* **599**, 623–638
- Weiss, L., Löffler, G., Shirmann, A. & Wieland, O. H. (1971) *FEBS Lett.* **15**, 229–231
- Whitehouse, S., Cooper, R. H. & Randle, P. J. (1974) *Biochem. J.* **141**, 761–774
- Yeaman, S. J., Hutcheson, E. T., Roche, T. E., Pettit, F. H., Brown, J. R., Reed, L. J., Watson, D. C. & Dixon, G. H. (1978) *Biochemistry* **17**, 2364–2370