Effect of micromolar concentrations of free Ca^{2+} ions on pyruvate dehydrogenase interconversion in intact rat heart mitochondria

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1. The mitochondrial content of active (dephospho) pyruvate dehydrogenase (PDH_A) was found to be severalfold higher at an extramitochondrial Ca^{2+} concentration of $1\ \mu\text{M}$ (pCa 6) than at pCa 7. The nature of the respiratory substrate did not affect this finding. 2. This Ca^{2+} -dependence was shown in state-4 and 50%-state-3 conditions [see Chance & Williams (1956) *Adv. Enzymol.* 17, 65–134], but was absent in the presence of excess ADP (state 3). 3. Na^+ and Mg^{2+} ions shifted the pCa value required for a maximal PDH_A content to lower values. This was attributed to a stimulation of mitochondrial Ca^{2+} egress and an inhibition of uptake, respectively. Na^+ ions diminished pyruvate dehydrogenase phosphate phosphatase activity in mitochondria which had been extensively depleted of Ca^{2+} ions by incubation with EGTA, raising the possibility of a direct inhibitory effect of Na^+ ions, unrelated to Ca^{2+} movements. 4. Mg^{2+} ions lowered the mitochondrial PDH_A content at pCa 6.24 and 6.48, but had only minimal effects in the presence of EGTA. 5. The effects of P_i and bicarbonate ions on PDH_A content were also studied, as possible effectors of mitochondrial Ca^{2+} transport. Bicarbonate ions abolished the response to Ca^{2+} ions, by generating maximal values of PDH_A content, but such a response was still observed when physiological concentrations of both P_i and bicarbonate were used. 6. The pCa of the medium in the range 6.33 to over 7 affected PDH_A content, with only very minor changes in state-4 rates of O_2 uptake and no change in $[\text{ATP}]/[\text{ADP}]$ ratio or in mitochondrial $[\text{NADH}]/[\text{NAD}^+]$ ratio, provided that Mg^{2+} ions were present. Thus the effect of Ca^{2+} ions on PDH_A content is unlikely to be mediated by changes in $[\text{ATP}]/[\text{ADP}]$ and $[\text{NADH}]/[\text{NAD}^+]$ ratio and is more likely to be direct. Equally, changes in the $[\text{acetyl-CoA}]/[\text{CoA}]$ ratio in response to Ca^{2+} ions when the substrate was pyruvate were the converse of those required to mediate changes in interconversion, and are probably secondary to changes in PDH_A content.

Ca^{2+} ions in the micromolar concentration range have been shown to facilitate the binding of the pyruvate dehydrogenase phosphate phosphatase to the transacetylase core of the pyruvate dehydrogenase complex (Denton *et al.*, 1972; Pettit *et al.*, 1972; Randle *et al.*, 1974; Severson *et al.*, 1974). This promotes the dephosphorylation of the α subunit of enzyme 1 of the complex (Linn *et al.*, 1969*a,b*; Yeaman *et al.*, 1978), generating an increased amount of the catalytically active dephospho form, PDH_A . Experiments with isolated mitochondria have also shown that addition of

Abbreviation used: PDH_A , active (dephospho) pyruvate dehydrogenase.

Ca^{2+} ions in the presence of the bivalent-cation ionophore A23187 leads to an enhanced content of PDH_A (Severson *et al.*, 1974; Walajtys *et al.*, 1974; Chiang & Sacktor, 1975). Equally, a mobilization of mitochondrial Ca^{2+} ions as a response to the addition of insulin to adipose tissue is an attractive possibility for the mechanism of action of that hormone in increasing adipose-tissue PDH_A content (Severson *et al.*, 1974, 1976; Denton *et al.*, 1978*a*). In muscles, increased availability of Ca^{2+} ions to the mitochondria during contraction may reasonably be expected to lead to an increased steady-state content of Ca^{2+} in the mitochondrial matrix and a consequent stimulation of pyruvate

dehydrogenase phosphatase activity. This would lead to an increased PDH_A content and would reinforce the activation seen on enhancement of myofibrillar ATPase activity and thought to be mediated via a decline in the mitochondrial $[\text{ATP}]/[\text{ADP}]$ and $[\text{NADH}]/[\text{NAD}^+]$ ratios (see Hansford, 1980a, for a review).

A previous report from our laboratory (Hansford & Cohen, 1978) showed that the PDH_A content of intact respiring heart mitochondria could be manipulated by the use of Ca^{2+} -EGTA buffers, with a large decrement in PDH_A content occurring when the pCa was raised from 6.2 to 6.8. In contrast with previous studies (Severson *et al.*, 1974; Walajtys *et al.*, 1974), Hansford & Cohen (1978) did not use a bivalent-cation ionophore, raising the possibility that this effect might be of physiological importance; consistent with this was the finding of a rapid reversibility.

The purpose of the present study was to define the conditions of substrate and ADP availability that allow an effect of Ca^{2+} ions to be seen, and to go on to see whether PDH_A content faithfully responds to experimental parameters expected to manipulate the steady-state mitochondrial-matrix Ca^{2+} concentration. These included the concentrations of Mg^{2+} and phosphate ions, which affect the electrophoretic uniport uptake system for Ca^{2+} ions [Crompton *et al.* (1976a); see Bygrave (1978) and Carafoli (1979) for reviews], and the concentration of Na^+ ions, which affects the charge-compensated egress system (Crompton *et al.*, 1976b).

Portions of this work have been presented as preliminary communications (Hansford, 1979, 1980b).

Experimental

Mitochondria were prepared from the hearts of male Wistar-derived rats, as described previously (Hansford, 1978). They were incubated at 25°C in media described for the appropriate Table or Figure. O_2 uptake was recorded in every incubation with a Clark-type O_2 electrode (Yellow Springs, OH, U.S.A.) in a 2 ml glass vessel (Gilson). At the appropriate time, $50\mu\text{l}$ samples of the incubation were removed and added to $250\mu\text{l}$ portions of an ice-cold stopping solution, of composition described previously (Hansford, 1976, 1977). The stopping solution is designed to lyse the mitochondria and prevent any further pyruvate dehydrogenase interconversion. Pyruvate dehydrogenase was then assayed spectrophotometrically, under V_{max} conditions, as described previously (Hansford, 1976). This activity is referred to as ' PDH_A content' in the text.

Mitochondrial Ca^{2+} uptake was measured by

using a Ca^{2+} electrode (Radiometer, no. F2002) and a Beckman Expandomatic pH-meter.

Mitochondrial Ca^{2+} content was measured by atomic-absorption spectroscopy (Perkin-Elmer no. 306 instrument) after sedimenting the mitochondria for 4 min in an Eppendorf Microfuge and extracting the mitochondrial pellet in 1% (v/v) $\text{HCl}/1\%$ (w/v) LaCl_3 .

Mitochondrial NADH content was measured with a Farrand fluorimeter (model A3) with primary filter no. 7-37 and secondary filters nos. 4-70 and 3-73. It should be noted that the fluorescence measured in these studies is due to NADPH as well as NADH. However, NADP is essentially completely reduced in coupled mitochondria and thus any differences in fluorescence may be attributed to differences in NADH content (see, e.g., La Noue *et al.*, 1970). Mitochondrial CoA and acetyl-CoA contents were determined in neutralized HClO_4 extracts, exactly as described previously (Hansford, 1976).

Ca^{2+} -EGTA buffers were made as described by Portzehl *et al.* (1964). In experiments in which Ca^{2+} -EGTA buffers were used, the pH of the incubation medium and of all additions was meticulously adjusted to 7.20.

Nagarse was obtained from Enzyme Development Corp., New York, NY, U.S.A., and other enzymes were from Boehringer Mannheim Corp., New York, NY, U.S.A. All reagents were of the highest grade commercially available and were made up in deionized and glass-distilled water.

Results and discussion

Minimum requirements for the demonstration of a Ca^{2+} effect on PDH_A content

The previous paper (Hansford & Cohen, 1978) had used a complex medium containing pyruvate plus palmitoylcarnitine as respiratory substrate, and glucose, ATP, MgCl_2 and hexokinase to generate a respiratory rate of 50% state 3 [see Chance & Williams (1956) for terminology]. Under these conditions, micromolar concentrations of free Ca^{2+} ions modulated PDH_A content. Fig. 1 shows that glutamate plus malate can be substituted as respiratory substrate and allow a dependence of PDH_A content on the pCa of the mitochondrial medium. Fig. 1 presents an abbreviated time-course of pyruvate dehydrogenase interconversion: early time-points are omitted, as they relate only to the low-energy and non-reproducible status of the mitochondria on storage at 0°C . Either the 5 min or 10 min points could be justified as giving near-steady-state values for PDH_A content. The increase in PDH_A from 5 to 10 min evident at pCa 6.33 and 6.48 is not understood and was not seen in an analogous study using a low concentration of

Fig. 1. Time-course of inactivation of pyruvate dehydrogenase in heart mitochondria oxidizing glutamate plus malate

Mitochondria (1.5–2 mg of protein) were added to 2 ml of a medium comprising 0.11 M-KCl, 20 mM-Hepes (K⁺ salt), 20 mM-potassium phosphate, 10 mM-NaCl, 5 mM-L-malate/Tris, 5 mM-L-glutamate/Tris and 1 mM-MgCl₂. The final pH was 7.2. Each incubation contained in addition a Ca²⁺-EGTA buffer of the pCa value indicated and of concentration 2 mM (as EGTA). At the times shown, samples of the incubations were withdrawn and quenched, as described in the Experimental section, and assayed for PDH_A activity. The values given are means $\pm$ S.E.M. for three separate experiments, except for the case where pCa was 7.26, for which experiments were in duplicate only and errors are not presented. They are presented as percentage of maximal PDH_A content, this being determined by sampling at 5 min in a medium containing 5 mM-pyruvate, 2.5 mM-malate, 5 mM-ADP and no added Ca²⁺ or Na⁺ ions. Maximal PDH_A values were in the range 98–135 nmol/min per mg of protein.

pyruvate (100 μ M) plus malate as substrate (results not shown). Despite this, the majority of the work described in this paper used glutamate plus malate, to avoid problems of pyruvate depletion. The time adopted for sampling was 10 min.

Other experiments indicated that whereas the mitochondrial PDH_A content was responsive to pCa when the mitochondria were respiring in state 4 (Fig. 1) and 50% state 3 (Hansford & Cohen, 1978), there was no such response in state 3. Thus, PDH_A content was measured as 80 nmol/min per mg of protein both at pCa 6.24 and in the presence of 2 mM-EGTA. The first two states are characterized

by limited ADP availability, the last by excess of ADP (see Chance & Williams, 1956). In this experiment, low concentrations of pyruvate were used (either none or 50 μ M) to avoid inhibition of the pyruvate dehydrogenase kinase by pyruvate (Hucho *et al.*, 1972). In addition, state 3 was elicited by addition of hexokinase just sufficient to generate a maximal rate of O₂ uptake: under these conditions the mean [ATP]/[ADP] ratio of the suspension was 3.1. Lower [ATP]/[ADP] ratios, as achieved in the simple addition of ADP to a mitochondrial suspension, would be more likely to inhibit the kinase (Hucho *et al.*, 1972) and thus even

Fig. 2. Effect of Na^+ ions on the Ca^{2+} -dependence of PDH_A content of state-4 mitochondria

Mitochondria (1.5–2 mg of protein) were added to 2 ml portions of each of five media containing 0.1–0.12 M-KCl, 20 mM-Hepes (K^+ salt), 20 mM-potassium phosphate, 5 mM-L-glutamate, 5 mM-malate, 2 mM- Ca^{2+} -EGTA buffer (as EGTA), 1 mM- MgCl_2 and NaCl at the concentrations indicated: $\bullet$, 0; $\blacksquare$, 0.1 mM; $\circ$, 1.0 mM; $\square$, 10 mM; $\blacktriangle$, 20 mM. The sum of the NaCl and KCl concentrations was constant, and the pH was 7.2. Sampling for PDH_A content was at 10 min. Points are mean values $\pm$ S.E.M. from four experiments, involving at least two different mitochondrial preparations, and data have been normalized on the basis of the maximal PDH_A content of each preparation, as described for Fig. 1. This value was 120 ± 7 nmol/min per mg for the six preparations used. Points without error bars are means of two determinations only.

less likely to reveal inactivation in the presence of EGTA. It would appear that modulation of pyruvate dehydrogenase interconversion by Ca^{2+} ions is ineffective in the presence of low $[\text{ATP}]/[\text{ADP}]$ (and $[\text{NADH}]/[\text{NAD}^+]$; see Hansford, 1980a) ratios in heart mitochondria. This does not mean that the binding of the pyruvate dehydrogenase phosphatase to the transacetylase is no longer sensitive to Ca^{2+} ions (see Denton *et al.*, 1972; Pettit *et al.*, 1972; Randle *et al.*, 1974; Severson *et al.*, 1974) under these conditions, but solely that the interconversion system is already poised heavily in favour of the phosphatase by virtue of the magnitude of the two ratios mentioned above.

Ca^{2+} -dependence of PDH_A content, and the effect of Na^+ ions

Fig. 2 presents the results of experiments like those of Fig. 1 but carried out at a variety of Na^+

concentrations and sampled for PDH_A at 10 min. It shows that the effect of Na^+ ions is to lower the PDH_A content at any particular value of pCa, such that the pCa needed to give a 50% activation of pyruvate dehydrogenase is shifted to lower values with increasing Na^+ concentrations, being pCa 6.79 at 0 mM- Na^+ and 6.74, 6.58, 6.39 and 6.32 at 0.1 mM-, 1 mM-, 10 mM- and 20 mM- Na^+ respectively. From these results, the Na^+ concentration required to give a half-maximal effect is found to be approx. 1.5 mM.

The effect of Na^+ ions can be explained by the finding that in heart mitochondria the release of Ca^{2+} ions is thought to involve a $\text{Ca}^{2+}/n\text{Na}^+$ exchange, where n is either 2 or 3 (Crompton *et al.*, 1976b; Crompton & Heid, 1978). Availability of the co-substrate for transport might be expected to enhance Ca^{2+} egress relative to its entry, and thus to lower the concentration of Ca^{2+} ions in the mitochondrial matrix, this being a steady-state value determined by the rate of transport in both directions (see Nicholls, 1978). This in turn would give rise to a lesser pyruvate dehydrogenase phosphatase activity (Denton *et al.*, 1972; Pettit *et al.*, 1972; Randle *et al.*, 1974; Severson *et al.*, 1974), assuming that the enzyme is localized in the matrix. This is reasonable in view of the necessity for pyruvate to cross the mitochondrial inner membrane to be oxidized by pyruvate dehydrogenase (Halestrap & Denton, 1975) and the response of PDH_A content to changes in matrix $[\text{NADH}]/[\text{NAD}^+]$ and $[\text{acetyl-CoA}]/[\text{CoA}]$ ratios (Hansford, 1976). Comparison of Fig. 2 here with Fig. 1 of Hansford & Cohen (1978) shows a reasonably close correspondence in pCa value needed to give a PDH_A value of 50%, i.e. 6.69 and 6.56 respectively at 1 mM- Na^+ . This is despite differences in respiratory substrate and metabolic state. Other Na^+ concentrations were not used previously.

Figs. 3(a) and 3(b) show the kinetics of inactivation of pyruvate dehydrogenase after the addition of EGTA to a mitochondrial suspension; inactivation (reflecting an excess of kinase over phosphatase activity) occurs much more rapidly in the presence of 20 mM- Na^+ than in the presence of 1 mM ($t_{1/2}$ = approx. 40 s and 3 min respectively). It is noted that buffers of different pCa values were used in the preliminary portions of these experiments, to match PDH_A content as nearly as possible at the point of addition of EGTA, making the protein substrate concentrations for the kinase and phosphatase similar in each experiment. A consequence of this is that the pCa after the addition of EGTA also differs slightly (see legend to Fig. 3) in Figs. 3(a) and 3(b), but is probably high enough to minimize Ca^{2+} uptake in both cases. This experiment is entirely consistent with the Na^+ ion facilitating Ca^{2+} egress when EGTA is added, and with the lowered matrix

Fig. 3. *Effect of Na⁺ ions on the rate of pyruvate dehydrogenase inactivation induced by the addition of EGTA* Mitochondria (3.3 mg of protein) were added to 2 ml of medium comprising 0.1 M-KCl, 20 mM-NaCl, 20 mM-Hepes (K⁺ salt), 20 mM-potassium phosphate, 0.7 mM-MgCl₂ and 2 mM-Ca²⁺-EGTA buffer, pCa 6.05 (a), or 0.12 M-KCl, 1 mM-NaCl, 20 mM-Hepes (K⁺ salt), 20 mM-potassium phosphate, 0.9 mM-MgCl₂ and 2 mM-Ca²⁺-EGTA buffer, pCa 6.48 (b). In both cases, the medium also contained 5 mM-L-glutamate and 5 mM-L-malate and was of pH 7.2. After sampling for PDH_A at 5 min, 10 μ mol of EGTA was added, and sampling was continued at the times shown. The calculated pCa values for this phase of the experiment were 7.53 and 7.61 for (a) and (b) respectively. The experiments were carried out twice, and the data are mean values. In the experiments marked (O), no EGTA was added.

Ca²⁺ concentration decreasing the activity of the pyruvate dehydrogenase phosphatase (Denton *et al.*, 1972; Pettit *et al.*, 1972; Severson *et al.*, 1974). Direct measurements of Ca²⁺ movement were made with a Ca²⁺ electrode and support the view that Na⁺ ions promote egress. These results are not presented, as they are repetitive of the work of Crompton *et al.* (1976b).

The effect of Na⁺ ions in promoting the rate of pyruvate dehydrogenase inactivation on addition of EGTA (Fig. 3) tends to confirm the steady-state nature of the experiments in Fig. 2, i.e. that Na⁺ ions are acting on the egress of Ca²⁺ ions in that system. However, inspection of Fig. 2 shows that PDH_A content varies with Na⁺ concentration, even in the presence of EGTA. This made it seem possible that Na⁺ ions were acting directly on the kinase or phosphatase, as well as affecting the movement of Ca²⁺ ions. To investigate which enzyme was the locus of action, the experiments in Fig. 4 were performed. Addition of 10 mM-dichloroacetate to the steady state would be expected to give a very large degree of inhibition of the kinase (Whitehouse *et al.*,

1974; Hansford & Cohen, 1978). The resulting increase in PDH_A content (Fig. 4) reflects excess phosphatase activity and is more rapid in the absence of Na⁺ ions. This implicates a direct inhibition of the phosphatase by Na⁺ ions. An experiment of analogous design, in which KF was added to 5 mM to inhibit phosphatase activity, and thus reveal the activity of the kinase, gave rates of pyruvate dehydrogenase inactivation which were essentially the same at 0 and 20 mM-Na⁺, in the presence of EGTA and 0.15 mM-dichloroacetate (added to inhibit the kinase partially and thus generate a high PDH_A content during the preincubation phase of the experiment). PDH_A content declined from 90 to 47 nmol/min per mg in 6.5 min from the addition of KF in the former case and from 76 to 30 nmol/min per mg in the latter: the time-courses are superimposable. Thus there is no evidence for a direct effect of Na⁺ ions on the kinase.

The results shown in Fig. 4 can, however, also be interpreted on the basis of a less than complete depletion of mitochondrial Ca²⁺ ions by EGTA

Fig. 4. Effect of Na^+ ions on the rate of re-activation of pyruvate dehydrogenase on addition of dichloroacetate in a Ca^{2+} -free medium

Mitochondria (3.4 mg of protein) were added to 2 ml of a medium comprising either 0.1 M-KCl, 20 mM-NaCl, 20 mM-Hepes (K^+ salt), 20 mM-potassium phosphate, 5 mM-L-glutamate, 5 mM-L-malate, 2 mM-EGTA and 2.5 mM- MgCl_2 (a) or an identical medium in which KCl replaced NaCl (b); 6.5 min later, dichloroacetate (DCA) was added to 10 mM final concn. and the incubation was sampled for PDH_A at the times indicated. The open circles represent control experiments in which medium was added instead of dichloroacetate. The experiments were performed with two mitochondrial preparations, and the points are the mean values. The mean maximal PDH_A content was 99 nmol/min per mg of protein.

in the absence of Na^+ . For this reason, samples were taken at the point of addition of dichloroacetate in Fig. 4, and assayed for Ca^{2+} by atomic-absorption spectroscopy. The mitochondrial Ca^{2+} content was 0.76 ± 0.11 nmol/mg of protein in the incubation without Na^+ and 0.42 ± 0.048 nmol/mg in the incubation containing 20 mM- Na^+ . Results are mean values $\pm$ S.E.M., from four experiments on two mitochondrial preparations and are significantly different ($P = 0.025\text{--}0.05$). Thus, although these Ca^{2+} contents are extremely low, the fact that they are different gives currency to the idea that Na^+ ions act entirely through their effect on Ca^{2+} transport. Experiments of the design of Fig. 4 but with a 13 min preincubation period still show differences in phosphatase activity, however, so that a direct effect of Na^+ ions on the phosphatase has not been ruled out absolutely.

Effect of Mg^{2+} ions on PDH_A content

The concentration of free Mg^{2+} ions might be expected to interact with the effect of Ca^{2+} ions on mitochondrial PDH_A content, in that Mg^{2+} ions have been shown to inhibit the electrophoretic uniport of Ca^{2+} , especially in heart mitochondria (Sordahl, 1974; Jacobus *et al.*, 1975; Crompton *et al.*, 1976a). From the foregoing, this would be expected to lower PDH_A content. At the same time, an elevated matrix content of Mg^{2+} ions will favour phosphatase over kinase activity, in view of the

differential in their K_m values for Mg^{2+} ions, yielding an increased PDH_A content (Linn *et al.*, 1969a; Hucho, 1974). Which effect predominates will depend on the degree of penetration of Mg^{2+} ions into the heart mitochondria. Unfortunately, a very relevant study in which these relations were examined used exclusively freeze-thawed heart mitochondria, with presumably impaired permeability properties (Schuster & Olson, 1974). For this reason, the question was re-examined.

Fig. 5 shows that PDH_A content is maximal at pCa 6.5 in the absence of Mg^{2+} ions and in the presence of 10 mM- Na^+ . Inspection of Fig. 2 shows that in the presence of 1.0 mM- MgCl_2 the maximum is found instead at pCa 6.05. Statistical analysis reveals that PDH_A content is less at pCa 6.05 and greater at pCa 6.48, in the absence of Mg^{2+} ions, with $P < 0.01$ in each case. The same shift in Ca^{2+} -dependency owing to the presence of Mg^{2+} ions is seen when results obtained at 1 mM- Na^+ are compared (cf. Figs. 2 and 5). This result is entirely consistent with the inhibition of Ca^{2+} uptake by Mg^{2+} ions, and a correspondingly decreased matrix concentration of free Ca^{2+} ions. It is admitted that state-4 rates of O_2 uptake decrease with pCa throughout the range of Ca^{2+} -EGTA buffers when no Mg^{2+} ions are present (see Expt. a of Table 2). Under these conditions, intramitochondrial $[\text{ATP}]/[\text{ADP}]$ and $[\text{NADH}]/[\text{NAD}^+]$ ratios may rise appreciably, affecting PDH_A content by well-

described mechanisms (see Hansford, 1980a, for a review). To avoid complications of this sort, most of the studies described in the present paper were

performed in the presence of Mg²⁺ ions. Under these conditions, there are wide ranges of pCa under which mitochondrial energy status is essentially invariant with pCa (see below).

The marked decrease in PDH_A content at lower pCa values seen in Fig. 5 is attributed to a massive uptake of Ca²⁺ ions and is thought to reflect mitochondrial pathology rather than physiology. It is associated with elevated state-4 rates of O₂ uptake (Table 2, Expt. a) which increase with time, mitochondrial [NADH]/[NAD⁺] ratios which decline progressively and diminished [ATP]/[ADP] ratios (results not shown). This phase is notably absent from Fig. 2, which uses a MgCl₂ concentration of 1 mM, providing a free [Mg²⁺] close to that thought to exist *in vivo* (see below).

Data presented in Table 1 show the dependence of PDH_A content on the concentrations of added MgCl₂, at two different values of pCa. PDH_A content declines with Mg²⁺ concentration, reaching minimal values at 0.9 mM-MgCl₂, when the pCa is 6.48. At pCa 6.24, somewhat higher concentrations of Mg²⁺ ions are required to give this decrement in PDH_A content, consistent with the mechanism of action of the Mg²⁺ ion being the inhibition of Ca²⁺ uptake by the electrophoretic uniport system

Fig. 5. Dependence of mitochondrial PDH_A content on pCa in the absence of Mg²⁺ ions
Conditions were as for Fig. 2, except that MgCl₂ was omitted. Media contained 10 mM-NaCl (□) or 1 mM-NaCl (○). Results presented are mean values ± s.e.m., normalized on the basis of maximal PDH_A content (see Fig. 1). This value was 104 ± 7 nmol/min per mg of protein.

Table 1. Effect of Mg²⁺ ions on PDH_A content of state-4 mitochondria in the presence (a) and absence (b) of Ca²⁺ ions
(a) Mitochondria (2.5–3 mg of protein) were added to 2 ml of medium comprising 0.11 M-KCl, 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; K⁺ salt], 20 mM-potassium phosphate, 10 mM-NaCl, 5 mM-L-glutamate, 5 mM-L-malate, 2 mM-Ca²⁺-EGTA buffer (as EGTA), and the concentration of MgCl₂ shown. The pH was 7.2. In (b), the medium used was the same as for (a), except where phosphate was omitted, in which case KCl was present at 0.14 M. Instead of a Ca²⁺-EGTA buffer, EGTA was added to 0.25 mM. In both (a) and (b), sampling for PDH_A content was at 10 min. The values given are means ± s.e.m. for either four (a) or three (b) experiments involving two mitochondrial preparations. They are presented as percentage of maximal PDH_A content (see legend to Fig. 1). Rates of O₂ uptake are presented as means ± s.e.m., with the numbers of mitochondrial preparations in parentheses: in (b) data are mean values for two preparations.

	[MgCl ₂] (mM)	PDH _A content (% of max.)	State-4 O ₂ uptake (ng-atoms of O/min per mg)
(a)			
pCa 6.24	0	75.5 ± 4.0	46.4 ± 3.0 (4)
	0.25	65.6 ± 4.4	—
	0.5	54.8 ± 2.1	37.5 ± 1.6 (4)
	0.9	49.9 ± 2.9	—
	2.5	41.8 ± 0.5	37.9 ± 1.3 (4)
pCa 6.48	0	64.4 ± 6.9	45.4 ± 1.6 (3)
	0.13	52.1 ± 4.8	—
	0.25	40.3 ± 3.0	—
	0.5	34.5 ± 2.5	35.3 ± 1.2 (3)
	0.9	29.4 ± 0.8	—
	2.5	29.7 ± 1.9	33.9 ± 0.9 (3)
(b) EGTA (0.25 mM)			
No P _i	0	89 ± 5	22.6
	1	90 ± 2	19.5
	2.5	99 ± 2	19.2
20 mM-P _i	0	46.8 ± 2.0	36.4
	1	45.0 ± 1.8	27.5
	2.5	47.2 ± 1.8	28.0
	5	47.5 ± 1.2	28.4

(Sordahl, 1974; Jacobus *et al.*, 1975; Crompton *et al.*, 1976a). When Ca^{2+} ions are not present (Table 1b), added MgCl_2 either has no effect on PDH_A content, or causes it to increase slightly, in the absence of P_i ($P = 0.05$ for the comparison of 0 and 2.5 mM- MgCl_2). This small effect suggests Mg^{2+} transport into the mitochondrial matrix and a direct effect on pyruvate dehydrogenase interconversion. These results are quite different from those of Schuster & Olson (1974), who found that Mg^{2+} ions increased PDH_A content markedly, especially in the presence of P_i . This probably reflected loss of Mg^{2+} ions on freeze-thawing and repletion by magnesium phosphate uptake during the course of the experiment.

Effect of phosphate, acetate and bicarbonate ions on the dependence of PDH_A content on pCa

Acetate, bicarbonate and phosphate ions cause the acidification of the mitochondrial matrix (see Chappell & Crofts, 1966), and this may oppose Ca^{2+} efflux, owing to the operation of Na^+/H^+ (Mitchell & Moyle, 1967; Brierley, 1976) and $\text{Ca}^{2+}/2\text{Na}^+$ (Crompton *et al.*, 1976b) exchange processes. In addition, these anions balance the electrophoretic uptake of Ca^{2+} ions driven by respiration (see Lehninger *et al.*, 1967, for a review). Thus they are expected to stabilize an enhanced matrix Ca^{2+} content. For this reason their effect on the pCa-dependence of PDH_A content was studied. It was found that PDH_A content was maximal over the range pCa 6.05–6.9 in the presence of 20 mM-acetate, during state-4 oxidation of glutamate plus malate, and near maximal in the presence of 20 mM-bicarbonate. However, in both cases values were also near maximal in the presence of EGTA, suggesting that these anions may also have effects unrelated to Ca^{2+} transport; with acetate, this could reflect inhibition of the kinase at the pyruvate site (Cooper *et al.*, 1974). The effect of P_i was found to be complex, with 2 mM- P_i giving decreased values for PDH_A content and 20 mM- P_i giving enhanced values, relative to incubations with only Cl^- as anion. For instance, at pCa 6.05 PDH_A values were 65, 39 and 74 nmol/min per mg for 0, 2 and 20 mM- P_i respectively. P_i has been shown to inhibit the purified pyruvate dehydrogenase phosphatase (Hucho *et al.*, 1972), and so these results cannot be interpreted solely in terms of mitochondrial Ca^{2+} transport. For this reason, the data are not presented more extensively. However, since P_i and bicarbonate both have large effects on PDH_A content, the latter by itself making it insensitive to pCa, it seemed important to ascertain whether there is indeed any dependence of PDH_A content on pCa at physiological concentrations of these anions. Fig. 6 shows experiments in which this was studied, by using a medium contain-

Fig. 6. Dependence of PDH_A content on pCa in the presence of both 15 mM-potassium phosphate and 10 mM- KHCO_3 .

Mitochondria (3 mg of protein) were added to 2 ml of a medium comprising 0.1 M-KCl, 20 mM-Hepes (K^+ salt), pH 7.2, 10 mM-NaCl, 15 mM-potassium phosphate, pH 7.2, 10 mM- KHCO_3 , 5 mM-L-glutamate/Tris, pH 7.2, 5 mM-L-malate/Tris, pH 7.2, 1 mM- MgCl_2 and the appropriate Ca^{2+} -EGTA buffer, present at 2 mM. The pH was adjusted to 7.20 with CO_2 immediately before each incubation. Where indicated, EGTA was present at 2 mM, with no added CaCl_2 . Incubations were sampled at 10 min for PDH_A content, and the values are mean values obtained from two mitochondrial preparations.

ing both 15 mM- P_i and 10 mM-bicarbonate; a dependence of PDH_A content on pCa, absent in the presence of bicarbonate alone (see above), is now restored. The shape of the dependence curve is only shifted slightly from the appropriate curve (10 mM-NaCl) in Fig. 2.

Controls on the effect of Ca^{2+} , Na^+ and Mg^{2+} ions on mitochondrial energy status

In discussing the effect of Ca^{2+} ions on pyruvate dehydrogenase interconversion, it is of paramount importance to separate direct effects of Ca^{2+} from effects that are mediated through changes in $[\text{ATP}]/[\text{ADP}]$ and $[\text{NADH}]/[\text{NAD}^+]$ ratios. Both of these affect interconversion (see Hansford, 1980a, for a review) and both are altered when mitochondria are exposed to more than micromolar concentrations of Ca^{2+} ions. Table 2 shows that state-4 rates of O_2 uptake are almost unchanged over the range pCa 6.05–6.90, in the presence of 10 mM- Na^+ and 1 mM- Mg^{2+} (Expt. b). This becomes clearer when they are compared with a state-3 rate

Table 2. *Effect of Ca²⁺ and Na⁺ ions on rates of O₂ uptake (a), the percentage reduction of mitochondrial NAD (b) and the mitochondrial [CoA]/[acetyl-CoA] ratio (c)*

In (a) and (b), the respiratory substrate was 5 mM-L-glutamate plus 5 mM-L-malate; in addition, media contained the components indicated in the Table, plus 20 mM-Hepes (K⁺ salt), pH 7.2, 20 mM-potassium phosphate, pH 7.2, and KCl to give a total osmolarity of 0.32. Ca²⁺-EGTA buffers were used at a concentration of 2 mM (as EGTA). O₂ uptake was followed as described in the Experimental section. The percentage reduction of mitochondrial NAD was determined by fluorimetry. Mitochondria were added to a medium without substrate and containing 0.25 mM-ADP. At 10 min, no further decline in fluorescence occurred, and this was taken as defining 0% reduction of NAD. After the addition of glutamate plus malate, the fluorescence in the state 4 after ADP phosphorylation was measured. This value is presented as a percentage of the span difference from 0 to 100% reduction, the latter being achieved by adding 1 mM-ADP and generating anaerobiosis. The numbers are mean values $\pm$ S.E.M., with the numbers of mitochondrial preparations in parentheses: where no error is given the number is the mean of two values. *P_i concentration was 2 mM. (c) For the measurement of the percentage reduction of NAD, the respiratory substrate was 50 μ M-pyruvate and 0.5 mM-L-malate; in addition, media contained 0.12 M-KCl, 20 mM-Hepes (K⁺ salt), 20 mM-potassium phosphate, 2 mM-Ca²⁺-EGTA buffer or EGTA, as appropriate, 1 mM-MgCl₂, and 2.5 mg of bovine serum albumin/ml. The Na⁺ concentration was 1.1 mM. The percentage reduction of NAD was measured essentially as for (a) and (b). For the determination of CoA and acetyl-CoA, the serum albumin was omitted and the concentration of pyruvate was 100 μ M. It was approximately maintained at this value by the addition of pyruvate, on the basis of O₂ uptake, as described by Hansford & Cohen (1978). Sampling was into HClO₄ at 5 min. The numbers are mean values $\pm$ S.E.M. with the numbers of determinations in parentheses. The CoA data were obtained from two mitochondrial preparations, and the NAD data from six.

				State-4 O ₂ uptake
(a) Experiment	pCa	[Mg ²⁺] (mM)	[Na ⁺] (mM)	(ng-atoms of O/min per mg)
a	6.05	0	10	53.0
a	6.33	0	10	42.0
a	6.60	0	10	40.3
a	6.90	0	10	37.0
a	7.26	0	10	34.4
b	6.05	1	10	31.5
b	6.33	1	10	29.9 $\pm$ 2.8 (3)
b	6.60	1	10	28.8 $\pm$ 2.1 (3)
b	6.90	1	10	27.7 $\pm$ 2.5 (3)
b	2 mM-EGTA	1	10	23.4
c	2 mM-EGTA	1	0	23.0 $\pm$ 0.4 (3)
c	2 mM-EGTA	1	1	24.5 $\pm$ 1.9 (3)
c	2 mM-EGTA	1	20	23.4 $\pm$ 0.4 (3)
d	0.5 mM-EGTA	0	0	39.4 $\pm$ 2.6 (6)
d	0.5 mM-EGTA	2.5	0	32.3 $\pm$ 2.7 (6)
(b) Experiment	pCa	[Mg ²⁺] (mM)	[Na ⁺] (mM)	Percentage reduction of NAD
e	6.33	1	0	69.0 $\pm$ 2.6 (3)
e	6.33	1	1	69.2 (2)
e	6.33	1	10	60.9 $\pm$ 4.8 (4)
e	6.33	1	20	62.2 $\pm$ 3.9 (4)
e*	6.33	1	20	62.2 $\pm$ 6.1 (3)
f	6.05	1	20	64.1 $\pm$ 5.1 (3)
f	2 mM-EGTA	1	20	58.6 $\pm$ 4.4 (3)
(c) Experiment	pCa	Percentage reduction of NAD	[CoA]/[acetyl-CoA] ratio	
g	EGTA	64.1 $\pm$ 3.5 (5)	1.56 $\pm$ 0.22 (6)	
	6.48	68.4 $\pm$ 1.0 (6)	0.48 $\pm$ 0.16 (6)	
	6.33	63.5 $\pm$ 3.6 (6)	0.63 $\pm$ 0.1 (4)	

in excess of 300 ng-atoms of O/min per mg. Thus changes in energy status are unlikely to underlie the changes in PDH_A content with pCa in the experiments of Figs. 1 and 2, from which these O₂-uptake data are derived. Equally, changes in Na⁺ concentration have no significant effect on state-4 rates of O₂ uptake (Table 2, Expt. c).

Measurement of mitochondrial NADH content

by fluorimetry indicates that it is unchanged with pCa over the range 6.05 to >8 (as achieved with EGTA alone) in the presence of 1 mM-MgCl₂ and 20 mM-NaCl, and with glutamate plus malate as respiratory substrate (Table 2, Expts. e and f), conditions that show substantial differences in PDH_A content (Fig. 2). Equally, mitochondrial NADH content is unchanged with pCa over the

range 6.33 to >8 , when the Na^+ concentration is 1.1 mM and the respiratory substrate is pyruvate plus malate (Table 2, Expt. g). The corresponding values for PDH_A content were found to be 27.4 ± 2.2 (4) % of the maximal PDH_A , in the presence of EGTA, and 56.7 ± 2.2 (4) %, at pCa 6.33. Moreover, the percentage reduction of mitochondrial NAD is insensitive to Na^+ concentration when this is varied over the range 0–20 mM (Table 2, Expt. e). Again, PDH_A content responds to Na^+ concentration in this range (Fig. 2). Finally, the percentage reduction of mitochondrial NAD is the same as 2 mM- and 20 mM- P_i (Table 2, Expt. e).

Measurement of ATP and ADP in a suspension of mitochondria oxidizing 100 μM -pyruvate showed an unchanged ATP/ADP ratio of 44 ± 5 over the range pCa 6.24–7.26, but a decreased ratio of 22 at pCa 6.05, in the presence of 1 mM- MgCl_2 and 1.1 mM- Na^+ . These results are consistent with the unchanged percentage reduction of NAD over the range pCa 6.33 to >8 , under otherwise similar experimental conditions (Table 2, Expt. g), in view of the near equilibrium between redox reactions and phosphorylation, especially in state 4 (Erecińska *et al.*, 1974). Thus, within the circumscribed ranges of pCa, Mg^{2+} and Na^+ concentrations described, changes in [ATP]/[ADP] and [NADH]/[NAD $^+$] ratios should not be a material factor in pyruvate dehydrogenase interconversion.

Omission of Mg^{2+} ions totally changes the picture, with state-4 rates which become more sensitive to pCa over the range 6.05–7.26 (Table 2, Expt. a) and NADH contents which are not stable with time in state 4 (results not shown). The decline in state-4 respiration with increasing Mg^{2+} concentration at two different values of pCa is shown in Table 1(a). Interestingly, Mg^{2+} ions also decrease O_2 uptake in the absence of Ca^{2+} ions (Table 1b), possibly by preventing the cycling of univalent cations (see Azzi & Azzone, 1967; Wehrle *et al.*, 1976).

Finally, Table 2(c) also shows that Ca^{2+} ions cause a decrease in the ratio [CoA]/[acetyl-CoA], which will tend to favour a decrease, rather than an increase, in PDH_A content (Pettit *et al.*, 1975; Cooper *et al.*, 1975; Hansford, 1976). It is suggested that the change in this ratio is a consequence of the enhanced PDH_A content and the fact that flux through the reaction cannot rise because of the limitation of electron transfer in state 4. The decreased [CoA]/[acetyl-CoA] ratio modulates the activity of the PDH_A in the mitochondrion, adjusting it down by end-product inhibition (Garland & Randle, 1964; Bremer, 1969; Tsai *et al.*, 1973) or by substrate limitation, to the flux allowed by the respiratory chain.

General discussion

Free Ca^{2+} ions in the range pCa 6–7 exert a major

control over the PDH_A content of heart mitochondria. It is emphasized that these concentrations apply to the surrounding medium, and that matrix Ca^{2+} concentrations are probably higher (see Nicholls, 1978, for a rationale). Indeed, very recent experiments by Williamson & Murphy (1980), using metallochromic indicators and a null-point technique, established a matrix Ca^{2+} concentration in the range 60–150 μM , in respiring liver mitochondria subjected to varying Ca^{2+} loads. However, it is hard to see how the Ca^{2+} concentrations could be this high in the matrix of respiring rat heart mitochondria, in view of the $K_{0.5}$ of approx. 1 μM for Ca^{2+} activation of isolated heart pyruvate dehydrogenase phosphatase (Denton *et al.*, 1972, 1975) and the responsiveness of pyruvate dehydrogenase interconversion to Ca^{2+} ions shown in respiring rat heart mitochondria (Hansford & Cohen, 1978; the present paper). Regardless of the mitochondrial-matrix Ca^{2+} concentration which is generated, the range pCa 6–7, which was established as crucial in the experiments *in vitro* described in the present paper, is precisely that to which several Ca^{2+} -dependent activities in the cytosol (Katz, 1970; Brostrom *et al.*, 1971) or outer face of the mitochondrial membrane (Hansford & Chappell, 1967) respond. Thus it is plausibly the concentration to which mitochondria are exposed *in vivo*.

The response to Ca^{2+} ions is greatly modified by the presence of Na^+ ions (Fig. 2). Although it is important to define Na^+ concentration as a condition that has to be tightly controlled in studies *in vitro*, it is not clear that it forms the basis of any metabolic control *in vivo*. This is because the response of PDH_A content to Na^+ ions is not very sensitive around 10 mM- Na^+ (Fig. 2), which is close to the cytosol concentration (Lee & Fozzard, 1975). Thus any response to the small influx of Na^+ ions on excitation may be slight.

The pronounced decrease in PDH_A content with Mg^{2+} concentration at any given value of pCa (Table 1, and cf. Figs. 2 and 5) is quite different from reports in the literature (Schuster & Olson, 1974) and probably relates to the great sensitivity to inhibition by Mg^{2+} ions of the Ca^{2+} -ion uniport system in heart mitochondria (Crompton *et al.*, 1976a). It is noteworthy that a free Mg^{2+} concentration of approx. 0.5 mM has a large but not maximal effect (Table 1). Cytosolic free Mg^{2+} concentrations are probably close to this value, on the basis of the aconitase equilibrium (Veloso *et al.*, 1973). Large variations with changes in work load are perhaps not to be expected, on the basis of minimal changes in adenine nucleotides (Neely *et al.*, 1972; Illingworth *et al.*, 1975), which are important chelator species. Thus, although Mg^{2+} concentration of the medium profoundly affects the response of the pyruvate dehydrogenase inter-

conversion system to extramitochondrial Ca²⁺ ions, a change in Mg²⁺ concentration is unlikely to be part of the mechanism of response to an altered work load on the heart. It is emphasized that these comments refer only to cytosolic Mg²⁺ ions. Intramitochondrial Mg²⁺ concentration may change more in response to work load and has in fact been implicated as a control parameter in the response of PDH_A to changes in work load, in a computer-simulation study (Kohn *et al.*, 1979).

Finally, it is hard to predict the effect on pyruvate dehydrogenase interconversion of the increase in P_i concentration seen with increased work load (Illingworth *et al.*, 1975; Nishiki *et al.*, 1978). This is because of the twofold effects of P_i on the system, and the correspondingly complicated concentration-dependence mentioned above. However, it is clear that the interconversion of pyruvate dehydrogenase in heart mitochondria retains sensitivity to pCa in the range 6–7 when the concentrations of both P_i and bicarbonate are near the physiological values (Fig. 6).

In addition to the pyruvate dehydrogenase complex, the NAD–isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex have been shown to be activated by Ca²⁺ ions, with K_{0.5} values for this ion of approx. 1 μM (Denton *et al.*, 1978b; McCormack & Denton, 1979). Based on these findings, these authors (McCormack & Denton, 1979; and see below) have cogently developed the point of view that mitochondrial Ca²⁺ concentration controls, among other effectors, the activities of pyruvate oxidation and the tricarboxylate cycle, via its effect on those three non-equilibrium enzyme reactions. As developed in some detail by Hansford (1980a), such activation at the dehydrogenase level confers the advantage that the redox span (ΔE_h) from the NAD⁺/NADH couple to the cytochrome a₃ Fe³⁺/Fe²⁺ couple diminishes less as a consequence of the enhancement of electron transfer by ADP than it would if no such direct activation of the dehydrogenase occurred. This allows for high rates of respiration and phosphorylation without decreases in the adenine nucleotide phosphorylation potential intolerable to ATP-requiring activities in the cell.

Note Added in Proof (Received 16 September 1980)

Since the first submission of this paper, work appeared by McCormack & Denton (1980) and Denton *et al.* (1980), in which the response of pyruvate dehydrogenase interconversion to micromolar concentrations of Ca²⁺ ions was monitored in intact heart and adipose-tissue mitochondria. Factors affecting Ca²⁺ ingress or egress were varied and their effects on interconversion described. In general, there is excellent agreement between their

work and that contained in the present paper. In addition, Denton *et al.* (1980) were able to infer the magnitude of the Ca²⁺ gradient across the mitochondrial membrane from a comparison of K_{0.5} values for Ca²⁺-activation of oxidation in intact mitochondria and of the activity of isolated 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase phosphatase.

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