Biochem. J. (1979) **180**, 533–544
Printed in Great Britain

The Effects of Calcium Ions and Adenine Nucleotides on the Activity of Pig Heart 2-Oxoglutarate Dehydrogenase Complex

By JAMES G. McCORMACK and RICHARD M. DENTON
Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

(*Received* 11 *December* 1978)

1. The effects of Ca^{2+} (mainly by using EGTA buffers), pH, ATP and ADP on the activity of the 2-oxoglutarate dehydrogenase complex from pig heart were explored. 2. Ca^{2+} (about 30μM) resulted in a decrease in the apparent K_m for 2-oxoglutarate from 2.1 to 0.16mM (at pH7) without altering the maximal velocity. At 0.1mM-oxoglutarate there was a 4–5-fold activation by Ca^{2+}, with an apparent K_m for Ca^{2+} of 1.2μM. A similar activation was also observed with Sr^{2+} (K_m 15.1μM), but not with Mn^{2+} or Mg^{2+}. 3. The K_m of the complex for oxoglutarate decreased markedly from pH7.4 to 6.6. The effects of Ca^{2+} remained evident over this pH range. 4. In the presence of Mg^{2+}, ATP resulted in a marked increase in the apparent K_m for oxoglutarate, whereas ADP greatly decreased this parameter. The concentrations of adenine nucleotide required for half-maximal effects were about 100μM in each case. 5. The effects of the adenine nucleotides and Ca^{2+} on the apparent K_m for oxoglutarate appeared to be essentially independent of each other, reversible, and demonstrable in the presence of end-product inhibition by NADH and succinyl-CoA (3-carboxypropionyl-CoA). Overall, a 350-fold change in K_m could be obtained. 6. Effects similar to those described above were also observed on the activity of 2-oxoglutarate dehydrogenase from rat heart and brown adipose tissue. 7. We discuss the mechanisms controlling this enzyme's activity and compare these regulatory features with those of NAD–isocitrate dehydrogenase and the pyruvate dehydrogenase system, which are also sensitive to Ca^{2+} and adenine nucleotides.

In a publication from this laboratory (Denton *et al.*, 1978), it was proposed that an increase in the intramitochondrial concentration of Ca^{2+} in the range 0.1–10μM may be a common mechanism for enhancing the rates of utilization of a number of respiratory fuels in mammalian tissues. The proposal was based on the observations that Ca^{2+} concentrations in this range greatly alter the activity of the enzymes which interconvert the active and inactive forms of pyruvate dehydrogenase, pyruvate dehydrogenase phosphate phosphatase (Denton *et al.*, 1972, 1975; Severson *et al.*, 1974) and pyruvate dehydrogenase kinase (Cooper *et al.*, 1974), and also the activity of NAD–isocitrate dehydrogenase (Denton *et al.*, 1978). In addition, evidence has come from other laboratories that suggests that increases in the Ca^{2+} concentration may enhance the rates of oxidation of β-hydroxybutyrate (Malmström & Carafoli, 1976), succinate (Ezawa & Ogata, 1977) and fatty acids (Otto & Ontko, 1978).

It seemed important to examine the possibility that other enzymes in the citrate cycle in addition to NAD–isocitrate dehydrogenase may be activated by Ca^{2+}. As will be reported in the present paper, only the activity of the 2-oxoglutarate dehydrogenase complex was found to be appreciably affected by Ca^{2+}; the K_m for 2-oxoglutarate was found to be decreased in the presence of μmolar concentrations of Ca^{2+}, by an order of magnitude. Oxoglutarate dehydrogenase catalyses a reaction with a large negative standard free-energy change, and it has been proposed on the basis of studies on the perfused heart (Randle *et al.*, 1970; Williamson *et al.*, 1973) and isolated heart mitochondria (Smith *et al.*, 1974) that it is an important site of regulation of the citrate cycle. The activity of mammalian oxoglutarate dehydrogenase is inhibited by its end products, succinyl-CoA (3-carboxypropionyl-CoA) and NADH (Garland, 1964; Smith *et al.*, 1974). Inhibition of the enzyme complex from insect flight muscle by $MgATP^{2-}$ and the reversal of this inhibition by ADP and AMP have been reported by Hansford (1972). Effects of adenine nucleotides on the mammalian complex do not appear to have been examined.

We have purified oxoglutarate dehydrogenase from pig heart mitochondria, and the present paper is mainly concerned with our detailed studies of the regulation of the enzyme by Ca^{2+} and adenine nucleotides.

Experimental

Chemicals and biochemicals

All coenzymes and substrates were from Boehringer Corp. (London) Ltd., Lewes, East Sussex BN7 2OF, U.K., and all other chemicals, including EGTA and EDTA, were the highest grade available from BDH Chemicals, Poole, Dorset, U.K., with the exception of Chelex 100 (100–200 mesh; Na^+ form), which was from Bio-Rad Laboratories, Watford, Herts., U.K.

Preparation of purified enzymes

The pyruvate dehydrogenase complex was prepared from pig heart as described by Cooper *et al.* (1974), by using a modification of the poly(ethylene glycol)-fractionation procedure of Linn *et al.* (1972). The same procedure was also employed in the preparation of the 2-oxoglutarate dehydrogenase complex up to the second poly(ethylene glycol) fractionation at pH 6.5. Oxglutarate dehydrogenase was precipitated with 1.5% (w/v) poly(ethylene glycol) and the pellet suspended in 50mM-Mops (4-morpholinepropanesulphonic acid), pH 7.0, containing 1mM-dithiothreitol to give about 10units/ml. The solution was centrifuged at 150000*g* for 90min and the pellet redissolved in Mops/dithiothreitol to give about 100units/ml and then stored in small samples at −20°C until use.

Preparation of mitochondrial extracts

Mitochondria were prepared from the interscapular brown adipose tissue of fed female rats (200–300g), which had been cold-adapted for 4–6 weeks at 5°C, by a slight modification of a method originally developed for epididymal white adipose tissue (Severson *et al.*, 1976). Mitochondria from rat hearts were prepared by the method of Kerbey *et al.* (1976). Samples of the intact mitochondria (2–4mg of protein) were then centrifuged in an Eppendorf 3200 minicentrifuge at about 10000*g* for 1min and subsequently extracted by freezing and thawing three times in 500μl of 100mM-KH_2PO_4 buffer (pH 7) containing 1mM-dithiothreitol and 50μl of rat serum/ml, the last being added to prevent proteolysis of the 2-oxoglutarate dehydrogenase complex (Linn, 1971; Wieland, 1975). The extract was centrifuged for 2min at 10000*g* and the supernatant used for the study of enzyme activities within 6h. No detectable 2-oxoglutarate dehydrogenase activity was removed by centrifugation under these conditions.

Sodium dodecyl sulphate/polyacrylamide gels

The preparation of the protein samples, the use of apparatus and the analysis of the resulting gels were essentially as described by Hughes & Denton (1976), except that a discontinuous gel system (Laemmli, 1970) was used.

Enzyme assays

Oxoglutarate dehydrogenase activity was measured by following the production of NADH at 340nm and 30°C by using a Gilford recording spectrophotometer (model 222). The assay medium was 50mM-Mops, pH 6.6–7.4 (as indicated), plus 1mM-dithiothreitol and containing appropriate additions of $MgCl_2$, $MnCl_2$, $SrCl_2$, $CaCl_2$, EGTA, EDTA, 2-oxoglutarate, NAD^+, thiamin pyrophosphate, CoA, ATP and ADP. Where required, Ca^{2+}-free buffer was prepared by passing the Mops/dithiothreitol buffer, pH 7.0, down a column of equilibrated Chelex 100 (100–200 mesh; Na^+ form) resin. Buffer used in the assays of mitochondrial extracts also contained 2μg of rotenone/ml in order to inhibit the NADH oxidase activity remaining in the extract (Denton *et al.*, 1978). Assays were conducted in a total volume of 1.5–1.7ml and were initiated by addition of purified enzyme or mitochondrial extract (2–10μl). Rates were essentially linear with time for at least 3–5min (end-product inhibition became evident after this) and the initial rates were also linear with amounts of enzyme or extract over the range used. Rates in the absence of added 2-oxoglutarate, NAD^+ or CoA were negligible.

All other enzymes were assayed in 50mM-Mops (pH 7) buffer plus 1mM-dithiothreitol and 1mM-$MgCl_2$ and with additions of appropriate substrates, cofactors and rotenone as required. Potassium salts were used throughout unless otherwise stated.

Handling of data

The use of EGTA buffers and calculation of free and bound concentrations of bivalent metal ions were as described by Denton *et al.* (1978). No allowance was made for the binding of the metal ions to NAD^+, 2-oxoglutarate or thiamin pyrophosphate. For NAD^+ and 2-oxoglutarate, binding is known to be comparatively weak (Colman, 1972; Sillén *et al.*, 1971) and will have negligible effects on free concentrations of the metal ions in the presence of 5mM-EGTA. We have been unable to find values of the stability constants for the binding of the bivalent metal ions to thiamin pyrophosphate. However, addition of thiamin pyrophosphate (1mM) had no appreciable effect on oxoglutarate dehydrogenase activity assayed under conditions of limiting Ca^{2+} concentrations provided that the Ca^{2+} was buffered with EGTA. This indicates that the Ca^{2+} concentration is little changed by the presence of thiamin pyrophosphate under these conditions. Apparent dissociation constants for the binding of the bivalent metal ions to EGTA and ADP were as given in Denton *et al.* (1978). The values used for MgATP and CaATP at pH 7.0 were 5.24×10^{-5}M and 1.32×10^{-4}M respectively calculated from stability constants given by Sillén *et al.* (1971).

Protein was measured by the method of Lowry *et al.* (1951), standardized by using bovine serine albumin. Enzyme activities are expressed as units of enzyme activity per mg of protein (where a unit of activity is taken as that amount of enzyme which transforms 1 μmol of substrate/min at 30°C) or as percentage of the maximal oxoglutarate dehydrogenase complex activity (measured at 25mM-oxoglutarate).

Kinetic constants were calculated by fitting data to the equations

$$v = V_{max.}/\{1+(K_m/[S])\}$$

and

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

by using a non-linear least-squares regression program written for a Hewlett Packard 9821 computer by Dr. Paul England of this department. However, n was found to be not significantly different from 1 in all cases and therefore the parameters quoted are derived from the first equation only.

Results and Discussion

Preliminary studies on the sensitivity of citrate-cycle and related enzymes to Ca^{2+}

As mentioned in the introduction, three mitochondrial enzymes have been shown to be sensitive to Ca^{2+} in the 0.1–10μM range. In our preliminary investigations we wished to ascertain whether this phenomenon was exhibited by any other enzymes of the citrate cycle or related mitochondrial pathways. We assayed the activity of the enzymes at two substrate concentrations, using mitochondrial extracts from rat heart and brown adipose tissue; one concentration was close to half the K_m of the particular enzyme for its substrate(s), and the other was saturating (i.e. to give the $V_{max.}$). The assays were performed with 1mM-$MgCl_2$ in the presence of either 5mM-EGTA (free Ca^{2+}<1nM) or 5mM-EGTA plus 5mM-$CaCl_2$ (free Ca^{2+} 20–33μM).

We could find no evidence that the catalytic activity of the following enzymes, at either high or low substrate concentrations, was affected by Ca^{2+} (up to 33μM): the pyruvate dehydrogenase complex, aconitate hydratase (EC 4.2.1.3), glutamate dehydrogenase (EC 1.4.1.2), malate dehydrogenase (EC 1.1.1.37), NADP–isocitrate dehydrogenase (EC 1.1.1.42) and succinate dehydrogenase (EC 1.3.99.1) (the last of these being assayed in a suspension of the membrane fraction of the extract). Succinate dehydrogenase was assayed in the presence and absence of 1mM-ATP and glutamate dehydrogenase in the presence and absence of ADP.

We found that 30μM-Ca^{2+} decreased the apparent K_m of citrate synthase (EC 4.1.3.7) for oxaloacetate from about 10 to 5μM in the absence of $MgCl_2$, without changing the $V_{max.}$. However, in the presence of 1mM-$MgCl_2$ the K_m was further decreased (to about 1–2μM) and the effect of Ca^{2+} was no longer detectable. It seems unlikely that this effect has physiological significance and thus we did not explore it any further.

The activity of the 2-oxoglutarate dehydrogenase complex was found to be stimulated about 4-fold by Ca^{2+} at a low 2-oxoglutarate concentration (0.1mM), yet the activity at saturating oxoglutarate (25mM) was unaffected by Ca^{2+}. This effect was evident in extracts of rat heart and brown-fat mitochondria and also in either the presence or absence of 1mM-$MgCl_2$, and indicated that probably the 2-oxoglutarate dehydrogenase complex was subject to control by Ca^{2+} in an analogous manner to the control by Ca^{2+} of NAD–isocitrate dehydrogenase (EC 1.1.1.41) (Denton *et al.*, 1978). In order to explore this further we examined the effects of Ca^{2+} on purified preparations of the enzyme from pig heart mitochondria.

Purity of oxoglutarate dehydrogenase prepared from pig heart

All the results reported in Figs. 1–7 and Tables 1–4 were obtained with a single preparation of 2-oxoglutarate dehydrogenase. Two other preparations of comparable purity were also studied and found to have very similar properties.

The particular preparation used had a specific activity of 10.6 units/mg of protein (at pH7), which is close to values published previously for the purified pig heart enzyme (Massey, 1960; Hayakawa *et al.*, 1964; Tanaka *et al.*, 1972). Protein components were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1). Three major protein bands were evident, of mol.wts. about 110000, 55000 and 45000, which correspond to the reported values for the oxoglutarate decarboxylase, dihydrolipoamide dehydrogenase and the dihydrolipoate succinyltransferase components of the complex respectively (Koike *et al.*, 1974). A number of minor bands of protein were also evident, with approximate mol.wts. of 130000, 35000, 18000 and 15000. It is not known whether all these represent impurities in the preparation. The preparation contained detectable activities of pyruvate dehydrogenase, NAD–isocitrate dehydrogenase and glutamate dehydrogenase; the maximum activities of these enzymes were 2.5, 0.7 and 3.0% respectively of the maximum activity of oxoglutarate dehydrogenase. The maximum activities of malate dehydrogenase and NADH oxidase were less than 0.3% of that of oxoglutarate dehydrogenase.

Effects of Ca^{2+} and calcium chelators on pig heart oxoglutarate dehydrogenase activity

Preliminary experiments showed that the expression of oxoglutarate dehydrogenase activity required

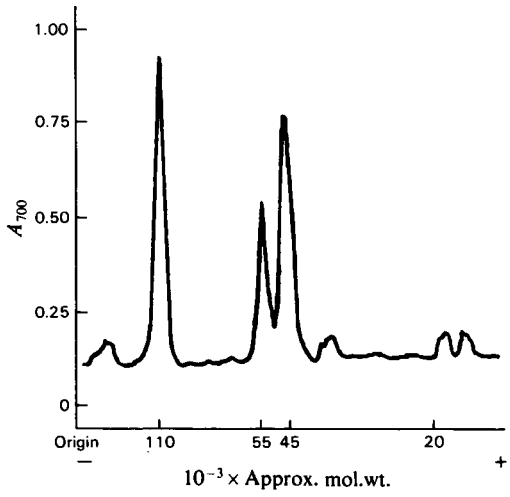

Fig. 1. *Densitometric trace of the protein components of purified oxoglutarate dehydrogenase preparation separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis*

Approximate values of the molecular weights of the major components are indicated. See the Experimental section for further details and references.

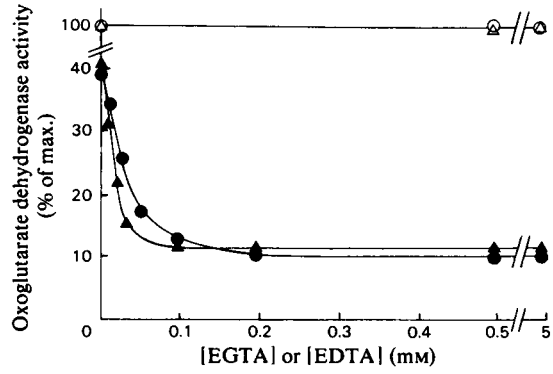

Fig. 2. *Effects of EGTA and EDTA on the activity of oxoglutarate dehydrogenase*

Activity was assayed at pH7 with various concentrations of EGTA (●, ○) or EDTA (▲, △) in the presence of 1mM-NAD^+, 1mM-thiamin pyrophosphate, 0.25mM-CoA and either 0.2mM-(●, ▲) or 25mM-(○, △) oxoglutarate: 100% activity corresponds to 10.1units/mg of protein. Each point shown is the mean of at least two observations. Similar results for EGTA were obtained in the presence of 1mM-$MgCl_2$.

NAD^+, CoA and oxoglutarate, but not added thiamin pyrophosphate or Mg^{2+}. Apparently thiamin pyrophosphate is so strongly bound to the pig heart enzyme that little or none is lost during the preparation and subsequent storage. In contrast, pig heart pyruvate dehydrogenase purified by a similar procedure shows only 2–30% of maximum activity in the absence of added thiamin pyrophosphate (Walsh *et al.*, 1976). There is also a marked difference in the requirement for Mg^{2+} between the two enzyme complexes. Pyruvate dehydrogenase activity requires Mg^{2+} with a reported K_m of about 5μM (Wieland *et al.*, 1969), and it has been shown that Mg^{2+} is required for the binding of thiamin pyrophosphate (Walsh *et al.*, 1976). In contrast, not only does pig heart oxoglutarate dehydrogenase not require added Mg^{2+}, but the addition of EDTA (up to 5mM) was found in the present study to have little or no effect on activity at saturating oxoglutarate concentrations (Fig. 2). The maximum activity of pyruvate dehydrogenase was inhibited by more than 98% by the addition of EDTA (5mM).

In the absence of added $CaCl_2$ or calcium chelators, the K_m for oxoglutarate of the purified pig heart oxoglutarate dehydrogenase was about 200μM, which is close to that found by previous workers (Hirashima *et al.*, 1967; Hamada *et al.*, 1975). Addition of EGTA or EDTA was associated with a marked decrease in the activity of oxoglutarate dehydrogenase at 0.2mM-oxoglutarate, but neither chelator had any effect on maximum activity measured at 25mM-oxoglutarate (Fig. 2). Half-maximal inhibition at 0.2mM-oxoglutarate was observed at about 50μM-EGTA or 20μM-EDTA. This is consistent with the removal of the endogenous Ca^{2+}, as EDTA binds Ca^{2+} more strongly than does EGTA. The sensitivity to EGTA is very similar to that observed previously with pyruvate dehydrogenase phosphate phosphatase (Randle *et al.*, 1974) and with NAD–isocitrate dehydrogenase (Denton *et al.*, 1978).

Fig. 3 illustrates the large effect of Ca^{2+} on the K_m for oxoglutarate. Activity at pH6.8 in the presence of 5mM-EGTA is compared with that in the presence of 5mM-EGTA plus 5mM-$CaCl_2$ (Ca^{2+} about 30μM). The values for K_m calculated from these data were 1.2mM in the presence of 5mM-EGTA and 150μM in the presence of 5mM-EGTA plus 5mM-$CaCl_2$, but the $V_{max.}$ was unaffected. Further evidence for the change in K_m for oxoglutarate with changing Ca^{2+} is given in Table 1. Calculated values of K_m and $V_{max.}$ are given for the enzyme assayed at pH7.0 and a series of Ca^{2+} concentrations obtained by using CaEGTA buffers. The K_m ranged from 2.5mM in the presence of less than 1nM-Ca^{2+} to 0.16mM in the presence of 33μM-Ca^{2+}, without any significant effect on the maximum activity. Under the same conditions, there was no change in the K_m for NAD^+ (the respective K_m values for NAD^+ in the presence of <1nM- and 33μM-Ca^{2+} were 61.2±4.7μM and 61.9±5.3μM; mean±S.D. for eight observations in each case).

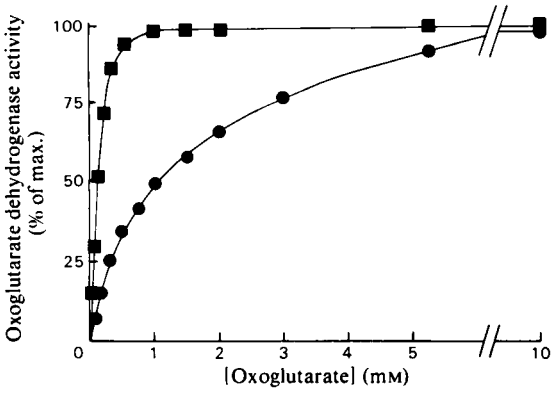

Fig. 3. *Effect of Ca^{2+} on the K_m of oxoglutarate dehydrogenase for oxoglutarate*

Activity was assayed at pH6.8 in the presence of 1mM-NAD^+, 1mM-thiamin pyrophosphate, 0.25mM-CoA, 1mM-$MgCl_2$, with oxoglutarate added as shown, and in the presence of 5mM-EGTA (●, Ca^{2+} <1nM) or 5mM-EGTA plus 5mM-$CaCl_2$ (■, Ca^{2+} about 30μM): 100% activity corresponds to 10.8 units/mg of protein. Each point shown is the mean of at least two observations.

Table 1. *Affinity of oxoglutarate dehydrogenase for oxoglutarate at various concentrations of Ca^{2+}*

Ca^{2+} concentrations were manipulated by using 5mM-CaEGTA buffers. Enzyme activity was assayed at pH7.0 in the presence of 1mM-NAD^+, 1mM-thiamin pyrophosphate, 0.25mM-CoA, 1mM-$MgCl_2$ and various concentrations of oxoglutarate. Kinetic constants are expressed as parameter ± S.D. (at least 10 degrees of freedom).

Calculated Ca^{2+} concn. (M)	Apparent K_m for oxoglutarate (mM)	$V_{max.}$ (units/mg of protein)
$<10^{-9}$	2.50±0.30	11.19±0.33
3.14×10^{-7}	0.98±0.11	10.49±0.25
5.01×10^{-7}	0.65±0.09	10.62±0.34
1.93×10^{-6}	0.31±0.04	10.46±0.27
3.27×10^{-5}	0.16±0.01	10.34±0.13

The inhibitory effects of 0.2mM-EGTA at low concentrations of oxoglutarate could be completely reversed by the subsequent addition of 5mM-EGTA plus 5mM-$CaCl_2$ or 5mM-EGTA plus 5mM-$SrCl_2$ or 25mM-oxoglutarate. The reversal appeared immediate (within the response time of the recording system of about 5–10s).

Effects of pH on the activity of pig heart oxoglutarate dehydrogenase

The maximum activity of oxoglutarate dehydrogenase was largely unaffected by changes in pH over the range 6.6–7.4 (Table 2). However, changes in the pH in this range greatly altered the K_m of the enzyme for oxoglutarate. In both the presence and the absence of Ca^{2+}, the activity of oxoglutarate dehydrogenase at 0.1mM-oxoglutarate was at least three times greater at pH6.6 than at pH7.4. In a separate experiment conducted in the presence of saturating Ca^{2+}, the K_m for oxoglutarate was 80μM at pH6.8, compared with 300μM at pH7.2. Nevertheless, clear effects of Ca^{2+} were observed throughout the pH range 6.6–7.4 (Table 2). Particular care was taken throughout this study that additions to the assay medium did not change the pH (within ±0.05unit). For example, in the experiments described in the following section ranges of free bivalent metal ion concentrations were required. These were generated by adding calculated amounts of stock solutions of EGTA (5mM) and EGTA (5mM) plus appropriate bivalent metal chloride (5mM), both adjusted to pH 7.0 (Denton *et al.*, 1978). This procedure minimizes the loss of protons which occurs when bivalent metal ions bind to EGTA at pH7.0.

Sensitivity of pig heart oxoglutarate dehydrogenase to Ca^{2+} and other bivalent metal ions

The results of a typical experiment using EGTA buffers to estimate the sensitivity of the oxoglutarate

Table 2. *Influence of pH on the activity of oxoglutarate dehydrogenase*

Activity was assayed in buffer at the pH indicated in the presence of 1mM-NAD^+, 1mM-thiamin pyrophosphate, 0.25mM-CoA and 1mM-$MgCl_2$ with additions of oxoglutarate, EGTA or $CaCl_2$ as indicated. The calculated Ca^{2+} concentrations in the presence of 5mM-EGTA plus 5mM-$CaCl_2$ were in the range 20–40μM. Values are the means of at least two observations.

	Oxoglutarate dehydrogenase activity (units/mg of protein) in			
	0.1mM-oxoglutarate with		25mM-oxoglutarate with	
pH	5mM-EGTA	5mM-EGTA plus 5mM-$CaCl_2$	5mM-EGTA	5mM-EGTA plus 5mM-$CaCl_2$
7.4	0.3	2.6	9.7	10.3
7.2	0.5	3.8	11.1	11.3
7.0	1.0	4.9	10.9	11.0
6.8	1.4	6.4	10.4	10.4
6.6	1.8	8.0	9.6	9.9

dehydrogenase to Ca^{2+}, Sr^{2+}, Mg^{2+} and Mn^{2+} at 0.1 mM-oxoglutarate are shown in Fig. 4. The apparent K_m for Ca^{2+} calculated from these data was $1.03 \pm 0.10\,\mu M$ (mean ± S.D. for 10 degrees of freedom). The maximum effect of Ca^{2+} was associated with a more than 5-fold increase in rate. A similar maximum effect could also be observed with Sr^{2+}, but a rather higher range of concentrations was necessary; the calculated K_m for Sr^{2+} from the data of Fig. 4 was $15.1 \pm 1.34\,\mu M$ (mean ± S.D. for 7 degrees of freedom). In contrast, Mg^{2+} and Mn^{2+} did not seem to have any activating effects. However, by using EGTA buffers it is not possible to explore the effects of Mn^{2+} beyond about $2.5\,\mu M$-Mn^{2+}, since above this concentration Mn^{2+} may displace Ca^{2+} from the EGTA (Denton *et al.*, 1978). Other studies into the sensitivity to Ca^{2+} showed that the K_m for Ca^{2+} at pH 7.0 was not altered by changes in oxoglutarate concentration. Thus the data of Table 1 gave values varying from 0.88 to $1.19\,\mu M$-Ca^{2+} for oxoglutarate concentrations which ranged from $25\,\mu M$ to 10 mM, the overall mean value being $0.97 \pm 0.05\,\mu M$. In a further separate experiment values of 0.97, 1.20, 1.04 and $0.94\,\mu M$ were obtained at oxoglutarate concentrations of 0.05, 0.1, 0.2 and 1 mM respectively (by using a range of eight different Ca^{2+} concentrations in each case). All these values were obtained by using EGTA–calcium buffers in the presence of 1 mM-thiamin pyrophosphate and 1 mM-$MgCl_2$; however, essentially identical values for K_m for Ca^{2+} were obtained by using the buffers in the absence of either or both thiamin pyrophosphate and $MgCl_2$.

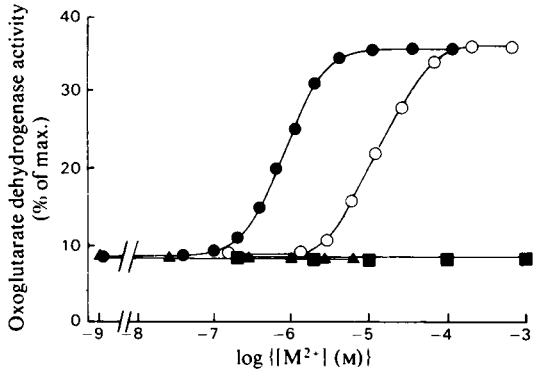

Fig. 4. *Effects of Ca^{2+}, Sr^{2+}, Mn^{2+} and Mg^{2+} on oxoglutarate dehydrogenase activity*
Activity was assayed at pH 7 with 5 mM-EGTA and additions of $CaCl_2$ (●), $SrCl_2$ (○), $MnCl_2$ (▲) and $MgCl_2$ (■) to give the indicated concentration of the appropriate metal ion (M^{2+}) in the presence of 1 mM-NAD^+, 1 mM-thiamin pyrophosphate, 0.25 mM-CoA and 0.1 mM-oxoglutarate: 100% activity corresponds to 10.2 units/mg of protein. Each point shown is the mean of at least two observations.

The effects observed with EGTA–calcium buffers could be the result, in part, of free EGTA, but not CaEGTA, having an inhibitory effect on the enzyme or of $CaCl_2$ displacing another metal ion from the EGTA, which in turn activates the enzyme rather than Ca^{2+}. We therefore carried out studies using assay buffer which had been freed of much of the endogenous Ca^{2+} by treatment with Chelex (Table 3). With treated buffer the activity at 0.1 mM-oxoglutarate was about half the activity in untreated buffer and approached the value observed with added EGTA or EDTA. Addition of $CaCl_2$ ($50\,\mu M$) or $SrCl_2$ ($500\,\mu M$) caused full activation, but no activation was apparent on addition of $MnCl_2$ (up to $100\,\mu M$) or $MgCl_2$ (up to 1 mM). These results confirm the results concluded from the experiments using EGTA buffers, namely that Ca^{2+} activates oxoglutarate dehydrogenase at low concentrations of oxoglutarate and that this effect is mimicked by Sr^{2+}, but not by Mg^{2+} or Mn^{2+}.

Table 3. *Sensitivity of oxoglutarate dehydrogenase to Ca^{2+}, Sr^{2+}, Mg^{2+} and Mn^{2+} in untreated and Ca-free buffers*
Activity was assayed at pH 7 in the presence of 1 mM-NAD^+, 0.25 mM-CoA and 0.1 mM-oxoglutarate and additions as indicated contained in either untreated or Chelex-treated buffer. Values shown are the means of at least two observations: 100% activity corresponds to 10.5 units/mg of protein.

Additions	Oxoglutarate dehydrogenase activity (% of max.) in Untreated buffer	Chelex-treated buffer
5 mM-EGTA	11.3	11.3
5 mM-EDTA	11.3	11.2
No addition	35.0	17.5
5 mM-EGTA plus 5 mM-$CaCl_2$	41.3	41.4
5 mM-EGTA plus 5 mM-$SrCl_2$	41.1	41.3
1 mM-$MgCl_2$	30.0	16.6
$100\,\mu M$-$MnCl_2$	31.3	17.1
$50\,\mu M$-$CaCl_2$	39.8	40.0
$500\,\mu M$-$SrCl_2$	41.3	40.0

Effects of ATP and ADP on the activity of pig heart oxoglutarate dehydrogenase

Initial experiments explored the effects of ATP and ADP, in the presence of $MgCl_2$, on the activity of oxoglutarate dehydrogenase assayed with oxoglutarate concentrations (2 and 0.1 mM) which gave about half-maximal activity at low (< 1 nM) and high (10–30 μM) Ca^{2+} concentrations respectively (Figs. 5*a* and 5*b*). The results indicated that ATP inhibited enzyme activity by up to 50% in both the presence and absence of saturating Ca^{2+} (half-maximal inhibition was observed at about 100 μM-ATP in each case). In contrast, ADP increased activity up to twofold under both conditions (half-maximal activation was observed at about 100 μM-ADP in each case). It should be noted that nucleotide concentrations were only taken up to 1.5 mM in the presence of Ca^{2+}, since above this concentration of either ATP or ADP the calculated Ca^{2+} concentration falls below 10 μM because of binding of Ca^{2+} to the nucleotide (Fig. 5*a*). Indeed marked inhibition was observed at ADP concentrations above 3 mM. This problem is not encounted at low Ca^{2+}, and no inhibition is seen at ADP concentrations up to 5 mM (Fig. 5*b*). Addition of increasing ADP in the presence of 1.5 mM-ATP under these conditions showed that ADP is able to re-activate the enzyme with a half-maximal stimulation at 400 μM, but there is still a clear effect of ATP at saturating ADP (Fig. 5*b*). AMP and GDP were also able to activate the enzyme, but the maximum effects were less than 20% of that of ADP and the concentrations required for half-maximal stimulation were at least 5 times greater. In mitochondria, the ATP and ADP concentrations do not change independently, as the total amount of adenine nucleotides remains constant. The effects of changes in the [ADP]/[ATP] ratio at a constant value of [ADP]+[ATP] (1.5 mM) are shown in Fig. 6. The largest effects are evident over values for the ratio in the range up to 0.3 in both the presence and absence of Ca^{2+}. Both ADP and ATP change the K_m of oxoglutarate dehydrogenase for oxoglutarate without altering the maximum activity (Table 4). Although Ca^{2+} also affects the K_m for oxoglutarate, regulation by Ca^{2+} and the adenine nucleotides appears to be essentially independent (Table 4). Overall, at constant pH, the K_m for oxoglutarate was found to vary over a 350-fold range from 0.07 mM in the presence of saturating ADP and Ca^{2+} to 24 mM in the presence of saturating ATP and Ca^{2+} below 1 nM.

It should be noted that there is no evidence that the effect of ATP is brought about by phosphorylation of oxoglutarate dehydrogenase. Studies on purified preparations of oxoglutarate dehydrogenase have shown no evidence of phosphorylation similar to that found with pyruvate dehydrogenase (Reed, 1974). Furthermore, when isolated mitochondria from rat heart, adipose tissue or other tissues are incubated with $^{32}P_i$ no incorporation of ^{32}P into any proteins of molecular weight corresponding to the components of the oxoglutarate dehydrogenase complex is detectable, although substantial incorporation is observed into the α-subunits of the pyruvate dehydro-

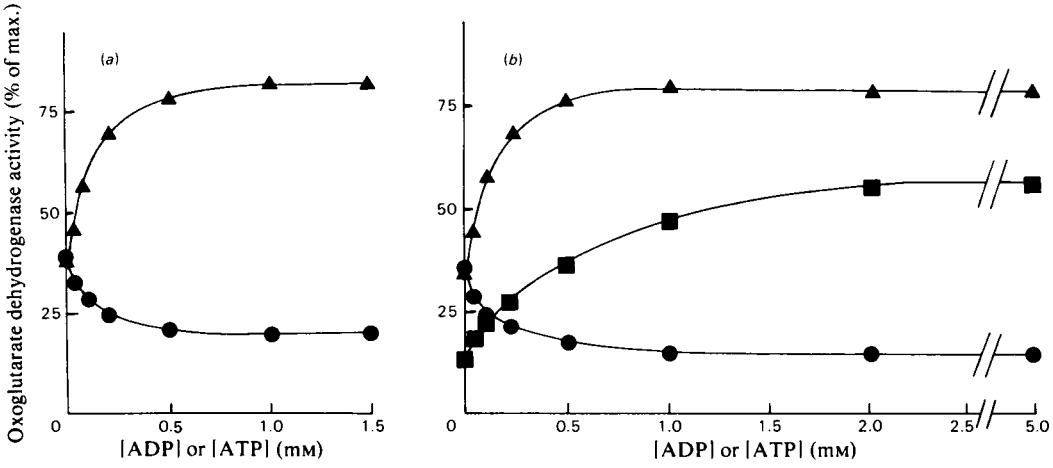

Fig. 5. *Effects of ATP and ADP on the activity of oxoglutarate dehydrogenase at (a) saturating Ca^{2+} (10–33 μM) and (b) minimal Ca^{2+} (< 1 nM)*

Activity was assayed at pH 7 with various concentrations of ATP (●), ADP (▲) or ADP with 1.5 mM-ATP (■), in the presence of 1 mM-NAD^+, 1 mM-thiamin pyrophosphate, 0.25 mM-CoA and 1 mM-$MgCl_2$ with the addition of either (*a*) 0.1 mM-oxoglutarate plus 5 mM-EGTA plus 5 mM-$CaCl_2$ or (*b*) 2 mM-oxoglutarate plus 5 mM-EGTA. In both (*a*) and (*b*) 100% activity corresponds to 9.8 units/mg of protein. Each point shown is the mean of at least two observations.

genase complex (Hughes & Denton, 1976). The concentrations of the two complexes in mammalian mitochondria are quite similar (Read *et al.*, 1977).

Effects of Ca^{2+} and adenine nucleotides under conditions of end-product inhibition of pig heart oxoglutarate dehydrogenase

Oxoglutarate dehydrogenase is inhibited by its end-products NADH and succinyl-CoA (Garland, 1964; Smith *et al.*, 1974). All the results presented so far have been on initial rates. To investigate whether Ca^{2+} and the adenine nucleotides have similar effects when the enzyme is inhibited by NADH and succinyl-

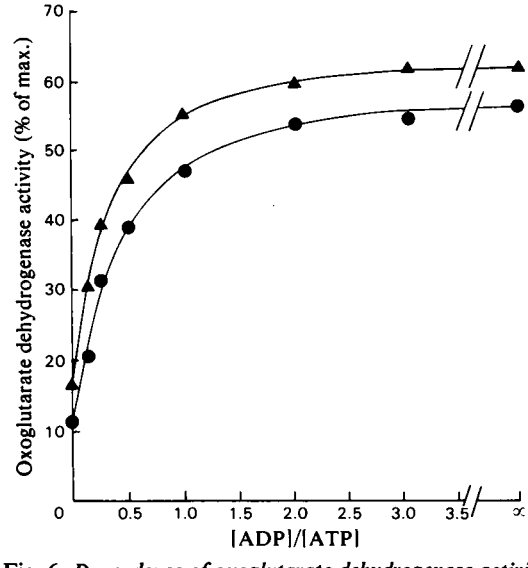

Fig. 6. *Dependence of oxoglutarate dehydrogenase activity on the ADP/ATP ratio*
Activity was assayed at pH 7 in the presence of 1 mM-NAD^+, 1 mM-thiamin pyrophosphate, 0.25 mM-CoA and 1 mM-$MgCl_2$ with additions of ADP and ATP to give the indicated ADP/ATP ratios in a total nucleotide concentration of 1.5 mM in the presence of either 0.1 mM-oxoglutarate plus 5 mM-EGTA plus 5 mM-$CaCl_2$ (Ca^{2+} 10–15 μM) (●) or 2 mM-oxoglutarate plus 5 mM-EGTA (Ca^{2+} < 1 nM) (▲): 100% activity corresponds to 10.5 units/mg of protein. Each point shown is the mean of at least two observations.

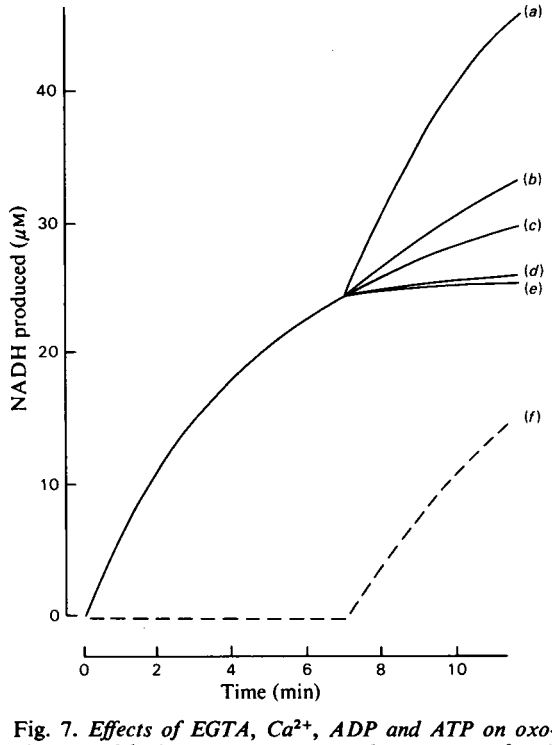

Fig. 7. *Effects of EGTA, Ca^{2+}, ADP and ATP on oxoglutarate dehydrogenase activity in the presence of end-product inhibition by NADH and succinyl-CoA*
Activity was assayed at pH 7 in the presence of 1 mM-NAD^+, 1 mM-thiamin pyrophosphate, 0.25 mM-CoA, 1 mM-$MgCl_2$ and initially (unbroken line) 0.1 mM-oxoglutarate or (broken line) no addition. Enzyme was added at zero time and the following additions were made at 7 min: (*a*) 1.5 mM-ADP, (*b*) 5 mM-EGTA plus 5 mM-$CaCl_2$, (*c*) no addition, (*d*) 1.5 mM-ATP, (*e*) 5 mM-EGTA and (*f*) 75 μM-oxoglutarate. The results of a typical experiment are shown.

Table 4. *Effects of Ca^{2+}, ADP and ATP on the kinetic constants for the oxoglutarate dehydrogenase reaction*
Activity was assayed at pH 7.2 in the presence of 1 mM-NAD^+, 1 mM-thiamin pyrophosphate, 0.25 mM-CoA, 1 mM-$MgCl_2$ and several oxoglutarate concentrations over a suitable range with additions of ATP, ADP, EGTA and $CaCl_2$ as indicated. Values are given as parameter ± S.D. (at least eight degrees of freedom).

Additions		$V_{max.}$ (units/mg of protein)	K_m for oxoglutarate (mM)
5 mM-EGTA (Ca^{2+} < 1 nM)	1.5 mM-ATP	11.2 ± 0.9	24.5 ± 4.2
plus	None	10.6 ± 0.8	8.6 ± 1.6
	1.5 mM-ADP	11.1 ± 0.4	0.84 ± 0.13
5 mM-EGTA plus 5 mM-$CaCl_2$	1.5 mM-ATP	10.7 ± 0.3	0.79 ± 0.10
(Ca^{2+} 10–33 μM) plus	None	10.7 ± 0.4	0.34 ± 0.05
	1.5 mM-ADP	10.5 ± 0.3	0.075 ± 0.011

CoA, assays with 0.1 mM-oxoglutarate were allowed to proceed until the concentration of oxoglutarate was decreased to 75 μM, while 25 μM-NADH and 25 μM-succinyl-CoA had accumulated (Fig. 7). Enzyme activity at this point was less than 50% of the initial rate obtained with 75 μM-oxoglutarate, thereby indicating the presence of end-product inhibition. Additions to give final concentrations of < 1 nM- and 30 μM-Ca^{2+}, 1.5 mM-ATP and 1.5 mM-ADP were then made. The results in Fig. 7 show that the effects of Ca^{2+}, ATP and ADP are all clearly exhibited and are of much the same magnitude as the effects seen in the absence of end-product inhibition.

Effects of Ca^{2+}, Sr^{2+}, ADP and ATP on the activity of oxoglutarate dehydrogenase in brown adipose tissue of cold-adapted rats

Since respiration in brown adipose tissue is not necessarily tightly coupled to the generation of ATP [see, e.g., Nicholls (1976) for review], it was of interest to see if oxoglutarate dehydrogenase from this tissue had similar properties to those found for the heart muscle enzyme, and in particular whether it was sensitive to ATP and ADP. The regulatory properties of oxoglutarate dehydrogenase in extracts of mitochondria prepared from the interscapular brown adipose tissue of cold-adaped rats were therefore investigated (Table 5). It was found that the properties of the rat brown-adipose-tissue enzyme were very similar to, if not identical with, those of the pig heart enzyme. At low concentrations of oxoglutarate, the activity was greatly increased by Ca^{2+} (or Sr^{2+}) and by ADP and decreased by ATP. These regulators had no effect on the maximum activity. Similar effects were also observed in mitochondrial extracts from heart of both control and cold-adapted rats.

General conclusions

The findings of the present paper reinforce our earlier proposal that changes in the mitochondrial concentration of Ca^{2+} may be an important means of regulating the rates of utilization of a number of respiratory substrates in mammalian tissues (Denton *et al.*, 1978). The K_m of oxoglutarate dehydrogenase for Ca^{2+} found in the present study was about 1 μM, and this value is very close to that of both NAD–isocitrate dehydrogenase (Denton *et al.*, 1978) and of the phosphatase (Denton *et al.*, 1972, 1975; Severson *et al.*, 1974) and kinase (Cooper *et al.*, 1974) involved in the interconversion of the active and inactive forms of pyruvate dehydrogenase (Denton *et al.*, 1975). Taken together, this represents strong support for the contention that the Ca^{2+} concentration in mitochondria may be 1 μM or less (Denton, 1977; Denton *et al.*, 1978). Nevertheless, more direct evidence that the intramitochondrial concentration

Table 5. *Effects of (i) Ca^{2+} and Sr^{2+} and (ii) ATP and ADP on oxoglutarate dehydrogenase activity in mitochondrial extracts of rat brown adipose tissue*

Activity was assayed at pH 7 in the presence of 1 mM-NAD^+, 1 mM-thiamin pyrophosphate, 0.25 mM-CoA and 1 mM-$MgCl_2$ with additions of oxoglutarate, EGTA, $CaCl_2$, $SrCl_2$, ATP and ADP as indicated. Results are expressed as % of the maximal oxoglutarate dehydrogenase activity [which was 191 ± 7 munits/mg of mitochondrial protein (mean ± S.E.M. for three observations on each of three different mitochondrial extracts)] and are given as means ± S.E.M. for the number of observations in parentheses, with each mean compiled from observations on at least three different extracts. *$P \leq 0.001$ compared with appropriate control value by Student's t test.

(i)

Addition	Oxoglutarate dehydrogenase activity (% of max.) in 0.1 mM-oxoglutarate	Oxoglutarate dehydrogenase activity (% of max.) in 25 mM-oxoglutarate
5 mM-EGTA (Ca^{2+} < 1 nM)	5.5 ± 0.3 (4)*	99.3 ± 1.3 (3)
None (control)	28.3 ± 1.0 (3)	100.2 ± 1.1 (3)
5 mM-EGTA plus 5 mM-$CaCl_2$ (Ca^{2+} approx. 30 μM)	39.8 ± 0.7 (3)*	101.5 ± 0.7 (3)
5 mM-EGTA plus 5 mM-$SrCl_2$ (Sr^{2+} approx. 500 μM)	40.1 ± 1.0 (4)*	99.4 ± 1.5 (3)

(ii)

Addition	Oxoglutarate dehydrogenase activity (% of max.) in 2 mM-oxoglutarate plus 5 mM-EGTA (Ca^{2+} < 1 nM)	Oxoglutarate dehydrogenase activity (% of max.) in 0.1 mM-oxoglutarate plus 5 mM-EGTA plus 5 mM-$CaCl_2$ (Ca^{2+} 10–30 μM)
1.5 mM-ATP	18.7 ± 1.4 (3)*	21.3 ± 0.9 (3)*
None (control)	40.3 ± 1.4 (6)	39.6 ± 1.4 (6)
1.5 mM-ADP	93.7 ± 3.9 (3)*	77.0 ± 2.1 (3)*

is in fact in this range is needed. It is possible that the Ca^{2+}-sensitive dehydrogenases present in mitochondria may prove to be a most useful means of monitoring intramitochondrial Ca^{2+} concentrations. For example, the apparent K_m of 2-oxoglutarate dehydrogenase for 2-oxoglutarate in isolated rat heart mitochondria has been estimated to be 0.7mM under conditions of a high intramitochondrial ADP/ATP concentration ratio (LaNoue *et al.*, 1973). This K_m value is considerably higher than that found in the present study for the purified pig or rat heart enzyme assayed in the presence of ADP and at saturating concentrations of Ca^{2+} (see Table 4); in fact, the value is consistent with there being an intramitochondrial Ca^{2+} concentration of less than 1μM under the conditions of the studies of LaNoue *et al.* (1973).

Changes in the intramitochondrial concentration of Ca^{2+} are likely to involve alterations in the activities of the processes transferring Ca^{2+} into and out of mitochondria. It is well established that mammalian mitochondria take up Ca^{2+} by a process driven by the membrane potential and which is inhibited by Ruthenium Red (see, e.g., Lehninger *et al.*, 1978; Nicholls, 1978). If the Ca^{2+} concentration in mitochondria is to be as low as 1μM, then this process must be offset by a second transporting system which can transfer Ca^{2+} back across the mitochondrial membrane (despite the membrane potential). This could be achieved if the charge on Ca^{2+} is compensated by the counter-transport of other cations, such as two or more Na^+ or H^+ ions (Puskin *et al.*, 1976; Pozzan *et al.*, 1977; Åkerman, 1978; Carafoli & Crompton, 1978) or by the simultaneous transport of an anion such as phosphate (Moyle & Mitchell, 1977) or phosphoenolpyruvate (Chudapongse & Haugaard, 1973; Peng *et al.*, 1974).

It is becoming evident that the Ca^{2+}-sensitivity of many cytoplasmic enzymes such as phosphodiesterase (Cheung, 1970, 1971), brain adenylate cyclase (Brostrom *et al.*, 1975; Cheung *et al.*, 1975), myosin light-chain kinase (Drabowska *et al.*, 1977; Yagi *et al.*, 1978) and phosphorylase *b* kinase (Cohen *et al.*, 1978) is brought about by binding of Ca^{2+} to the same protein, called the calcium-dependent regulator protein or calmodulin. This protein has a mol.wt. of 16700 (Lin *et al.*, 1974; Watterson *et al.*, 1976) and is closely related to the calcium-binding subunit of troponin (Stevens *et al.*, 1976; Watterson *et al.*, 1976). As we have stressed above, the sensitivity of pyruvate dehydrogenase phosphate phosphatase, NAD–isocitrate dehydrogenase and oxoglutarate dehydrogenase to Ca^{2+} is extremely similar. Furthermore, in all cases the effects of Ca^{2+} are also observed with Sr^{2+}, but not with Mg^{2+} and probably not Mn^{2+} (Severson *et al.*, 1974; Severson & Denton, 1979; Denton *et al.*, 1978; the present paper). This is consistent with the concept of a common calcium-binding protein being involved which remains bound to the enzymes during purification. Preparations of purified pyruvate dehydrogenase and oxoglutarate dehydrogenase (Fig. 1) do contain small amounts of protein components with mol.wts. in the range 15000–20000. However, no cytoplasmic calcium-dependent regulator protein was detected in these preparations [P. Cohen (Department of Biochemistry, University of Dundee), unpublished work], but the possibility that a related protein is involved has not been ruled out.

Clearly regulation by Ca^{2+} is not the only means whereby the flux through the pyruvate dehydrogenase system, NAD–isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex may be controlled (Table 6). The similarity of the factors con-

Table 6. *Summary of the factors regulating the activities of the pyruvate dehydrogenase system, NAD–isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex from mammalian heart*

Enzyme	Effects of increases in: Ca^{2+} (and Sr^{2+}) concentration	ADP/ATP concentration ratio	NAD^+/NADH concentration ratio
Pyruvate dehydrogenase system			
Catalytic activity of the non-phosphorylated complex	No effect (this paper)	No effect (R. M. Denton, unpublished work)	Activation (Garland & Randle, 1964)
Kinase activity	Inhibition (Cooper *et al.*, 1974)	Inhibition (Linn *et al.*, 1969)	Inhibition (Cooper *et al.*, 1975; Pettit *et al.*, 1975)
Phosphatase activity	Activation (Denton *et al.*, 1972)	No effect (Denton *et al.*, 1975)	No effect (Denton *et al.*, 1975)
Overall effect	Activation	Activation	Activation
NAD–isocitrate dehydrogenase activity	Activation (Denton *et al.*, 1978)	Activation (Chen & Plaut, 1963)	Activation (Chen & Plaut, 1963)
2-Oxoglutarate dehydrogenase complex activity	Activation (this paper)	Activation (this paper)	Activation (Garland, 1964)

trolling these three enzymes is very striking; all are stimulated not only by Ca^{2+} (and Sr^{2+}) but also by increases in the ADP/ATP and NAD^+/NADH concentration ratios. The flux through these three enzymes may be controlled in a concerted manner, and presumably the properties that they exhibit are important in muscle and other tissues to ensure that the rate through these dehydrogenases may be closely matched to cell ATP requirements.

We thank the Medical Research Council for financial support and for a studentship to J. G. McC. We are also grateful to Dr. P. J. England of this Department for making available the computer programs used in this study.

References

Åkerman, K. E. O. (1978) *Arch. Biochem. Biophys.* **189**, 256–262
Brostrom, C. O., Huang, Y. C., Breckenridge, B. M. & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 64–68
Carafoli, E. & Crompton, M. (1978) *Ann. N.Y. Acad. Sci.* **307**, 269–283
Chen, R. D. & Plaut, G. W. E. (1963) *Biochemistry* **2**, 1023–1032
Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun.* **38**, 533–538
Cheung, W. Y. (1971) *J. Biol. Chem.* **246**, 2859–2869
Cheung, W. Y., Bradham, L. S., Lynch, T. J., Lin, Y. M. & Tallow, E. A. (1975) *Biochem. Biophys. Res. Commun.* **66**, 1055–1062
Chudapongse, P. & Haugaard, N. (1973) *Biochim. Biophys. Acta* **307**, 599–606
Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. D. & Nairn, A. C. (1978) *FEBS Lett.* **92**, 287–293
Colman, R. (1972) *Anal. Biochem.* **46**, 358–363
Cooper, R. H., Randle, P. J. & Denton, R. M. (1974) *Biochem. J.* **143**, 625–641
Cooper, R. H., Randle, P. J. & Denton, R. M. (1975) *Nature (London)* **257**, 808–809
Denton, R. M. (1977) in *Hormones and Cell Regulation* (Dumont, J. & Nunez, J., eds.), vol. 1, pp. 167–170, North-Holland, Amsterdam
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
Drabowska, R., Aromatoril, O. D., Sherry, J. M. F. & Hartshorne, D. J. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1263–1272
Ezawa, I. & Ogata, E. (1977) *Eur. J. Biochem.* **77**, 427–435
Garland, P. B. (1964) *Biochem. J.* **92**, 10c–12c
Garland, P. B. & Randle, P. J. (1964) *Biochem. J.* **91**, 6c–7c
Hamada, M., Koike, K., Nakaula, Y., Hiraoka, T., Koike, M. & Hashimoto, T. (1975) *J. Biochem. (Tokyo)* **77**, 1047–1056
Hansford, R. G. (1972) *FEBS Lett.* **21**, 139–141
Hayakawa, T., Muta, H., Hirashima, M., Ide, S., Okabe, K. & Koike, M. (1964) *Biochem. Biophys. Res. Commun.* **17**, 51–56
Hirashima, M., Hayakawa, T. & Koike M. (1967) *J. Biol. Chem.* **242**, 902–907
Hughes, W. A. & Denton, R. M. (1976) *Nature (London)* **264**, 471–473
Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. & Denton, R. M. (1976) *Biochem. J.* **154**, 327–348
Koike, K., Hamada, M., Otsuka, K.-I., Suemastsu, T. & Koike, M. (1974) *J. Biol. Chem.* **248**, 3836–3842
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
LaNoue, K. F., Wałajtys, E. I. & Williamson, J. R. (1973) *J. Biol. Chem.* **248**, 7171–7183
Lehninger, A. L., Reynafarje, B., Vercesi, A. & Tew, W. P. (1978) *Ann. N.Y. Acad. Sci.* **307**, 160–174
Lin, Y. M., Liu, Y. P. & Cheung, W. Y. (1974) *J. Biol. Chem.* **249**, 4943–4954
Linn, T. C. (1971) *Arch. Biochem. Biophys.* **161**, 505–514
Linn, T. C., Pettit, F. H. & Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 234–241
Linn, T. C., Pelley, J. W., Pettit, F. H., Randall, D. D. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* **148**, 327–342
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Malmström, K. & Carafoli, E. (1976) *Biochem. Biophys. Res. Commun.* **69**, 658–664
Massey, V. (1960) *Biochim. Biophys. Acta* **38**, 447–460
Moyle, J. & Mitchell, P. (1977) *FEBS Lett.* **84**, 135–140
Nicholls, D. G. (1976) *Trends Biochem. Sci.* **1**, 128–130
Nicholls, D. G. (1978) *Biochem. J.* **176**, 463–474
Otto, D. A. & Ontko, J. A. (1978) *J. Biol. Chem.* **253**, 789–799
Peng, C. F., Prue, D. W., Bhuvaneswaran, C. & Wadkins, C. L. (1974) *Biochem. Biophys. Res. Commun.* **56**, 134–141
Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 575–582
Pozzan, T., Bragadin, M. & Azzone, G. F. (1977) *Biochemistry* **16**, 5618–5625
Puskin, J. S., Gunter, T. E., Gunter, K. K. & Russell, P. R. (1976) *Biochemistry* **15**, 3834–3842
Randle, P. J., England, P. J. & Denton, R. M. (1970) *Biochem. J.* **117**, 677–695
Randle, P. J., Denton R. M., Pask, H. T. & Severson, D. L. (1974) *Biochem. Soc. Symp.* **39**, 75–87
Read, G., Crabtree, B. & Smith, G. H. (1977) *Biochem. J.* **164**, 349–355
Reed, L. J. (1974) *Acc. Chem. Res.* **7**, 40–46
Severson, D. L. & Denton, R. M. (1979) in *Effects of Stable Strontium* (Skaryna, S. C., ed.), Ann Arbor Science Publishers, Ann Arbor, MI, in the press
Severson, D. L., Denton, R. M., Pask, H. T. & Randle, P. J. (1974) *Biochem. J.* **140**, 225–237
Severson, D. L., Denton, R. M., Bridges, B. J. & Randle, P. J. (1976) *Biochem. J.* **154**, 209–223
Sillén, L. G., Martell, A. E., Högfeldt, E. & Smith, R. M. (eds.) (1971) *Stability Constants: Supplement No. 1*, The Chemical Society, London
Smith, C. M., Bryła, J. & Williamson, J. R. (1974) *J. Biol. Chem.* **249**, 1497–1505
Stevens, F. C., Walsh, M., Ho, H. C., Teo, T. S. & Wang, J. H. (1976) *J. Biol. Chem.* **251**, 4495–4500

Tanaka, H., Koike, K., Hamada, M., Otsuka, K.-I., Suematsu, T. & Koike, M. (1972) *J. Biol. Chem.* **247**, 4043–4049

Walsh, D. A., Cooper, R. H., Denton, R. M., Bridges, B. J. & Randle, P. J. (1976) *Biochem. J.* **157**, 41–67

Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F. & Vanaman, T. C. (1976) *J. Biol. Chem.* **251**, 4501–4513

Wieland, O. H. (1975) *FEBS Lett.* **52**, 44–47

Wieland, O. H., Von Jagow-Westerman, B. & Stukowski, B. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 329–334

Williamson, J. R., Safer, B., LaNoue, K. F., Smith, C. M. & Wałajtys, E. (1973) *Symp. Soc. Exp. Biol.* **27**, 241–281

Yagi, K., Yazawa, M., Kakuichi, S., Oshino, M. & Uenishi, K. (1978) *J. Biol. Chem.* **253**, 1338–1340