Effect of micromolar concentrations of free calcium ions on the reduction of heart mitochondrial NAD(P) by 2-oxoglutarate

Richard G. HANSFORD and Frances CASTRO

Laboratory of Molecular Aging, National Institute on Aging, National Institutes of Health, Gerontology Research Center, Baltimore City Hospitals, Baltimore, MD 21224, U.S.A.

(Received 10 March 1981/Accepted 5 June 1981)

1. The reduction of mitochondrial NAD(P) by 2-oxoglutarate was monitored as a measure of 2-oxoglutarate dehydrogenase activity in its intramitochondrial locale. In the absence of ADP, steady-state reduction of NAD(P) by 0.5 mM-2-oxoglutarate in the presence of 0.5 mM-L-malate was markedly increased by extramitochondrial Ca^{2+} , with 50% activation at $\text{pCa} 6.58$, when the Na^+ concentration was 10 mM, the P_i concentration was 5 mM and the added Mg^{2+} concentration was 1 mM. Omission of P_i resulted in 50% activation at $\text{pCa} 6.77$; omission of Mg^{2+} resulted in 50% activation at $\text{pCa} \geq 7.3$. 2. The activation of 2-oxoglutarate dehydrogenase could be reversed on addition of an excess of EGTA. The rate of inactivation was dependent on the concentration of Na^+ , with $K_{0.5}$ 2.5 mM, which is consistent with the rate of withdrawal of Ca^{2+} from the mitochondria being the limiting factor. 3. The steady-state reduction of cytochrome *c* by 2-oxoglutarate (0.5 mM) also showed a marked dependence on pCa in the absence of ADP; in the presence of an excess of ADP, no such effect of Ca^{2+} was detectable. 4. Mitochondria from the hearts of senescent rats showed an undiminished rate of dehydrogenase activation by Ca^{2+} but a rate of inactivation by excess EGTA that was diminished by 40%. Direct studies of Ca^{2+} egress with Arsenazo III confirmed a decrement in rate with old age. 5. Studies of 2-oxoglutarate dehydrogenase activity as a function of the mitochondrial content of Ca^{2+} , as measured by atomic-absorption spectrophotometry, showed half-maximal activation at a mitochondrial content of 1.0 nmol of Ca^{2+} /mg of protein, and saturation at 3 nmol/mg. 6. These findings support the model advanced by Denton, Richards & Chin [(1978) *Biochem. J.* **176**, 899–906], of a control of the tricarboxylate cycle by intramitochondrial Ca^{2+} , and demonstrate the range of mitochondrial Ca^{2+} content over which this may occur. In addition, they raise the possibility of a disturbance of this control mechanism in old age.

McCormack & Denton (1979) have reported that Ca^{2+} activates 2-oxoglutarate dehydrogenase from heart tissue, apparently via an allosteric effect that leads to a lowering of the K_m of the enzyme for 2-oxoglutarate. A half-maximal effect was attained at approx. 1 μM free Ca^{2+} , in experiments involving the purified 2-oxoglutarate dehydrogenase complex. Further, Denton *et al.* (1980) have shown that the oxidation of 2-oxoglutarate by intact rat heart mitochondria is sensitive to extramitochondrial Ca^{2+} in the concentration range 20–300 nM, depending on Mg^{2+} and Na^+ concentrations, provided that non-saturating concentrations of 2-oxoglutarate are used. This latter study used State-3 rates of O_2 uptake (for terminology see Chance & Williams, 1956) as a

measure of 2-oxoglutarate oxidation and, by inference, of 2-oxoglutarate dehydrogenase activity. The present paper explores the effect of Ca^{2+} on the reduction of mitochondrial NAD(P) by 2-oxoglutarate and uses this as an index of dehydrogenase activity. This permits the study not only of 2-oxoglutarate dehydrogenase activity in the steady state but also of rates of activation and inactivation, which are not amenable to the oxygen-electrode technique. In addition, studies may be performed in State-4 respiration, where high $[\text{ATP}]/[\text{ADP}]$ ratios will make 2-oxoglutarate dehydrogenase particularly sensitive to Ca^{2+} , as Ca^{2+} and decreases in $[\text{ATP}]/[\text{ADP}]$ ratio both act by decreasing the K_m of 2-oxoglutarate dehydrogenase for its substrate

(McCormack & Denton, 1979). Effects of Na^+ , Mg^{2+} and P_i are described on the rate of enzyme inactivation seen on addition of EGTA to the mitochondrial suspension, and are partially interpreted in the light of the known kinetic properties of the $\text{Ca}^{2+}/n\text{Na}^+$ exchange responsible for the egress of Ca^{2+} from heart mitochondria (Crompton *et al.*, 1976b). This rate of inactivation is shown to be diminished in heart mitochondria isolated from senescent animals, raising the possibility of a looser linkage between contractile work and the control of the tricarboxylate cycle in old age.

In the steady state, measurement of the percentage reduction of cytochrome *c* as well as NAD(P) allows an estimate of the ΔE_h or redox span corresponding to two phosphorylation sites or loops of the respiratory chain (Mitchell, 1966). Activation of 2-oxoglutarate dehydrogenase by Ca^{2+} gives rise to an increase in this span in State-4 respiration but not in State 3, and this is discussed in connection with the adenine nucleotide phosphorylation potential that might be expected to be maintained.

Previous experiments in which activation of 2-oxoglutarate dehydrogenase (Denton *et al.*, 1980) or the pyruvate dehydrogenase system (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981) by Ca^{2+} has been studied with intact respiring mitochondria have used Ca^{2+} /EGTA buffers to stabilize low concentrations of free Ca^{2+} . The high total Ca^{2+} content of such buffers precludes the accurate measurement of intramitochondrial Ca^{2+} content, and this is not known in any case where dehydrogenase activation has been shown. The present study measures 2-oxoglutarate dehydrogenase activity and mitochondrial Ca^{2+} content in experiments in which the endogenous Ca^{2+} content of the mitochondria is decreased by small additions of EGTA and measured by atomic-absorption spectrophotometry. It is shown that the critical range of mitochondrial Ca^{2+} content is 0–3 nmol/mg of protein. This information is important in the light of a controversy over the notion advanced by Denton *et al.* (1978) that intramitochondrial free Ca^{2+} concentrations of about $1\text{ }\mu\text{M}$ control dehydrogenase activity. The controversy stems from direct measurements of matrix free Ca^{2+} concentrations in the range 60–150 μM (Williamson & Murphy, 1981), which appeared to be so high as to preclude a meaningful control of enzyme activity (Williamson & Cooper, 1980). However, these studies used mitochondrial Ca^{2+} loads in the range 10–36 nmol/mg of protein (Williamson & Murphy, 1981), much higher than the range of Ca^{2+} contents shown in the present study to allow modulation of dehydrogenase activity. Thus it seems that the enzymological results (Denton *et al.*, 1978; Denton & McCormack, 1980) and the measurements of free Ca^{2+} concentration (Williamson & Murphy, 1981)

may not be incompatible, but that the free Ca^{2+} concentration should be determined with Ca^{2+} loads of 3 nmol and less/mg of protein.

Experimental

Mitochondria were prepared from the hearts of male Wistar-derived rats of the ages stated, as described previously (Hansford, 1978). Digestion with Nagarse was rigidly standardized at 11 min; extension of this to 20 min caused a minor decrease in the rate of 2-oxoglutarate dehydrogenase inactivation by added EGTA. For experiments involving measurement of Ca^{2+} transport with Arsenazo III, and for the experiments on endogenous mitochondrial Ca^{2+} content, the mitochondria were given an additional wash with 8 ml of EGTA-free preparation medium.

Mitochondrial NAD(P)H content was measured by fluorimetry, and O_2 uptake with an oxygen electrode, as described by Hansford (1981). In addition, NADH and NADPH were measured separately in some experiments, in which mitochondrial incubations were sampled into ethanolic KOH and the nucleotides were assayed in neutralized extracts as described by Williamson & Corkey (1969), but with an Aminco–Chance DW-2A dual-wavelength spectrophotometer.

The reduction of cytochrome *c* was measured by using the wavelength pair 550 nm and 540 nm and an Aminco–Chance DW-2A dual-wavelength spectrophotometer. Calculations of E_h for the NAD^+ –NADH and cytochrome *c* Fe^{3+} – Fe^{2+} couples were made as described by Johnson & Hansford (1977).

Mitochondrial Ca^{2+} uptake was studied by using the metallochromic indicator Arsenazo III (Scarpa, 1979) and the dual-wavelength spectrophotometer at 575 nm and 650 nm. The protein content of the mitochondrial suspension was determined by the method of Murphy & Kies (1960), and the suspension was diluted to exactly 22.5 mg/ml. Of this, an 80 μl portion was added to 2 ml of medium containing 0.13 M-KCl, 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (potassium salt), 5 mM- P_i (potassium salt), 5 mM-L-glutamate, 5 mM-L-malate and 30 μM -Arsenazo III. After 5 min, CaCl_2 was added to give a concentration of between 2.5 and 37.5 μM . The rate of absorbance decrease corresponding to the withdrawal of this Ca^{2+} by the mitochondria was recorded. When uptake was complete, at 9 min after the addition of the mitochondria, NaCl (40 μmol) and Ruthenium Red (1.6 nmol) were added to initiate release, and the rate of release was recorded. All transport experiments were conducted at 25°C.

Mitochondrial Ca^{2+} content was measured by centrifuging a 1.5 ml portion of the mitochondrial

incubation for 4 min in an Eppendorf Microfuge, extracting the pellet in 1.5 ml of 1% (v/v) HCl/1% (w/v) LaCl₃ and measuring Ca²⁺ with a Perkin-Elmer 306 atomic-absorption spectrophotometer.

The $V_{\max}$ activity of 2-oxoglutarate dehydrogenase in suspensions of mitochondria from young and old rats was measured by disrupting the mitochondria with detergent and measuring the rate of reduction of exogenous NAD⁺ at 25°C by using a Gilford model 250 spectrophotometer. Mitochondria (0.4 mg of protein) were added to 2 ml of medium containing 50 mM-Hepes (potassium salt), pH 7.2, 1 mM-MgCl₂, 2.5 mM-dithiothreitol, 2.5 μ M-rotenone, 2 mM-KCN, 2.5 mM-NAD⁺, 0.22 mM-thiamin pyrophosphate, 60 μ M-CoA, 1 mM-Ca²⁺/EGTA, pCa 6.25, and 0.1% (v/v) Triton X-100. Then 2 min later the reaction was initiated by the addition of 2-oxoglutarate (20 μ mol). When the Ca²⁺-sensitivity of 2-oxoglutarate dehydrogenase was investigated as a function of animal senescence, the conditions used were the same, except that a range of buffers of different pCa values was used and the substrate was 0.25 mM-2-oxoglutarate.

Ca²⁺/EGTA buffers were made as described by Portzehl *et al.* (1964). An association constant of 1.204×10^7 at pH 7.2 was used. In experiments in which Ca²⁺/EGTA buffers were used, the pH of the incubation medium and of all additions was adjusted meticulously to 7.2.

Arsenazo III was type I, from Sigma Chemical Co. Ruthenium Red was a gift from Dr. J. Wehrle, Department of Physiological Chemistry, Johns Hopkins University, and had been recrystallized. All other reagents were of the highest grade available commercially and were made up in deionized and glass-distilled water.

Results and discussion

Effect of pCa on reduction of NAD(P) by 2-oxoglutarate

Fig. 1(a) shows that the addition of 2-oxoglutarate to a concentration of 0.5 mM causes very little reduction of NAD(P), provided that mitochondria have been depleted of endogenous Ca²⁺ by preincubation with EGTA, in the presence of Na⁺ (to allow Ca²⁺/nNa⁺ exchange; see Crompton *et al.*, 1976b). This is consistent with low 2-oxoglutarate dehydrogenase activity under these conditions. L-Malate is present at 0.5 mM to potentiate the transport of 2-oxoglutarate into the mitochondria (DeHaan & Tager, 1968; Chappell, 1968) and itself causes very little reduction. Addition of Ca²⁺/EGTA buffers then gives a marked enhancement in NAD(P)⁺ reduction, the degree of reduction achieved in the steady state being dependent on the pCa of the buffer. This is consistent with the activation of 2-oxoglutarate dehydrogenase by Ca²⁺,

Fig. 1. Effect of Ca²⁺ on the reduction of mitochondrial NAD(P) by 2-oxoglutarate

(a) Mitochondria (2 mg of protein) were added to 2 ml of medium containing 0.13 M-KCl, 20 mM-Hepes (potassium salt), 10 mM-NaCl, 5 mM-P_i (potassium salt), 1 mM-MgCl₂, 100 μ M-EGTA and 0.25 mM-ADP. The pH was 7.2 and the temperature 25°C. Subsequent additions were: 2-oxoglutarate (Ogl) (1 μ mol) and L-malate (Mal) (1 μ mol) at 6 min; Ca²⁺/EGTA buffers (3 μ mol of EGTA) of the pCa indicated, at 8.5 min; ADP (3 μ mol) at 10.5 min. The fluorescence of mitochondrial NAD(P)H was monitored as described in the Experimental section. (b) The early portion of the experiment was as described above, except that NaCl was omitted from the medium. The Ca²⁺/EGTA buffer was of pCa 6.64. At 3.5 min after the addition of the Ca²⁺/EGTA buffer, EGTA (4 μ mol) and NaCl (to give the concentrations shown, in mM) were added to initiate the oxidation of NAD(P)H.

at non-saturating concentrations of substrate, which was described by McCormack & Denton (1979). A much lesser effect of Ca²⁺ on NAD(P) reduction is seen when higher concentrations, e.g. 10 and 20 mM-2-oxoglutarate are used (results not shown). Shortly after the addition of Ca²⁺/EGTA buffer (Fig. 1a) the very small amount of ADP added at the beginning of the experiment to aid in the

oxidation of endogenous substrate has been phosphorylated, and the mitochondria are respiring in State 4. In Fig. 1(b) the steady state that follows Ca^{2+} /EGTA addition is then perturbed by the addition of EGTA, sufficient to raise pCa to 7.3 or above, and NaCl, to allow the egress of mitochondrial Ca^{2+} (Crompton *et al.*, 1976b; Crompton & Heid, 1978). It is seen that oxidation of NAD(P) occurs, the rate of which is dependent on the concentration of Na^+ present.

Sampling and alkaline extraction of the mitochondrial suspension in experiments of the type of Fig. 1(a) reveal an NADH content of 0.39 nmol/mg before the addition of Ca^{2+} /EGTA and of 2.38 nmol/mg in the steady state at pCa 6.64, in the absence of Na^+ . The latter conditions give an identical degree of fluorescence to that achieved at pCa 6.37 in Fig. 1(a) (where Na^+ concentration is 10 mM). By contrast, NADPH content increases only marginally on Ca^{2+} /EGTA addition, being 0.71 and 0.93 nmol/mg respectively. In experiments of the type of Fig. 1(b), the NADH content fell from 2.38 to 0.29 nmol/mg, when EGTA and NaCl were added (to give 10 mM- Na^+), and sampling was 5 min after the addition. By contrast, NADPH content was only slightly diminished, from 0.93 to 0.79 nmol/mg. All data for NADH and NADPH content are mean values from two separate experiments that differed by no more than 15%. Thus changes in the fluorescence of mitochondrial reduced nicotinamide nucleotides in response to the manipulation of mitochondrial Ca^{2+} content (Fig. 1) can be attributed very largely to changes in mitochondrial NADH content and are taken to represent this in the remainder of the present paper.

When rates from experiments of the type of Fig. 1(b) are plotted as a function of Na^+ concentrations (Fig. 2), a sigmoid curve is obtained, with $K_{0.5}$ 2.8 mM. Notably, direct determination of Ca^{2+} efflux gives a curve of similar shape, but with $K_{0.5}$ 8 mM (Crompton *et al.*, 1976b). The two experiments give different information, as it is possible that the Ca^{2+} concentration in the vicinity of the 2-oxoglutarate dehydrogenase complex is not the same as the mean value for the whole of the mitochondrial matrix space. Denton *et al.* (1980) reported $K_{0.5}$ values of 1.09 and 1.3 mM for an effect of Na^+ on rates of O_2 uptake with 2-oxoglutarate as substrate. The technique used in the present paper allows a more precise measure of the rate of inactivation of 2-oxoglutarate dehydrogenase by Ca^{2+} withdrawal to be made.

Fig. 2 also shows that the presence of Mg^{2+} results in an elevated rate of dehydrogenase inhibition on the addition of EGTA and NaCl. In these experiments Mg^{2+} was present from the beginning of the incubation; in other experiments (results not shown) MgCl_2 was added (to 1 mM) together with the EGTA and NaCl, and these experiments also

gave high rates of dehydrogenase inactivation. In addition, the presence of MgCl_2 abolished a lag phase of several seconds otherwise seen on addition of EGTA and NaCl. There is no obvious explanation of these results in terms of mitochondrial Ca^{2+} transport, as direct measurement of Ca^{2+} egress, by the Arsenazo III technique (see the Experimental section), gave $6.95 \pm 0.21(9)$ nmol/min per mg of protein in the absence of Mg^{2+} and $5.3 \pm 0.2(3)$ in the presence of 1 mM- MgCl_2 , when Ca^{2+} egress was initiated by the addition of Ruthenium Red and NaCl to mitochondria loaded with 11 nmol of Ca^{2+} /mg of protein, and MgCl_2 was added at the same time.

Omission of P_i results in much diminished rates of dehydrogenase inactivation (Fig. 2). This is a clear finding in that the steady-state extent of NAD(P) reduction before the addition of EGTA was the same in each case ($78.4 \pm 1.6\%$ reduced in the presence of P_i and $78.6 \pm 1.6\%$ in its absence). This tends to minimize the importance in this experiment of an

Fig. 2. Effects of Na^+ , Mg^{2+} and P_i on the rate of oxidation of mitochondrial NAD(P)H caused by the addition of EGTA

Rates of oxidation of mitochondrial NAD(P)H were derived from experiments performed as described for Fig. 1(b), but with higher recorder chart speeds where appropriate. In the experiments in which Mg^{2+} was omitted, the Ca^{2+} /EGTA buffer added in the first portion of the experiment was pCa 7.33, instead of pCa 6.64. Rates are expressed as percentages of the total NAD(P)H oxidized/min, where the total is proportional to the fluorescence obtained on anaerobiosis minus that obtained on substrate depletion before the addition of 2-oxoglutarate and malate. Results are mean values for two mitochondrial preparations differing by no more than 10%. ●, Complete system; ■, Mg^{2+} omitted; ○, P_i omitted.

effect of P_i on 2-oxoglutarate dehydrogenase activity via ΔpH , matrix 2-oxoglutarate concentration, mitochondrial $[ATP]/[ADP]$ ratio or the facilitation of the succinic thiokinase reaction. However, when transport was measured by the Arsenazo III technique, omission of P_i gave a slight enhancement in the rate of Ca^{2+} release $[10.0 \pm 0.3(3) \text{ nmol/min per mg from mitochondria loaded with } 11 \text{ nmol of } Ca^{2+}/\text{mg of protein in the absence of } P_i, \text{ versus } 6.95 \pm 0.21(9) \text{ for mitochondria with the same } Ca^{2+} \text{ load, but incubated with } 5 \text{ mM-} P_i \text{ throughout the experiment}]$. Thus again there is an apparent discrepancy; however, the experiments cannot be simply compared, as the Arsenazo III technique reveals the amount of Ca^{2+} released by the mitochondria, whereas the 2-oxoglutarate dehydrogenase responds presumably to the free Ca^{2+} concentration in the mitochondrial matrix, or a region thereof. These two parameters are related by the matrix buffering capacity for Ca^{2+} , which may not be the same in these experiments. Additionally, the mitochondrial Ca^{2+} loads are probably much less in the experiments involving $Ca^{2+}/EGTA$ buffers, where they may be inferred to be around 1 nmol/mg (see below).

The steady-state extents of NAD(P) reduction, obtained in experiments of the type of Fig. 1, are plotted in Fig. 3 as a function of pCa . When the Mg^{2+} concentration is 1 mM and the Na^+ concentration is 10 mM, conditions close to physiological (Veloso *et al.*, 1973; Lee & Fozzard, 1975), 50% activation of 2-oxoglutarate dehydrogenase occurs at pCa 6.58. When Mg^{2+} is omitted, activation occurs at much higher values of pCa (≥ 7.3). The difference is thought to reflect the effective inhibition of the electrogenic inward transport of Ca^{2+} by Mg^{2+} (Crompton *et al.*, 1976a), and has been discussed previously by Denton *et al.* (1980) and by Hansford (1981) in connection with the regulation of pyruvate dehydrogenase by Ca^{2+} . Omission of Na^+ also shifts the pCa required for 50% activation to higher values (results not shown), as expected from the similar effect seen when State-3 O_2 uptake with 2-oxoglutarate is measured (Denton *et al.*, 1980). Omission of P_i shifts the pCa for half-maximal activation to 6.77; this is consistent with the effects of P_i in stimulating dehydrogenase inactivation on the addition of EGTA discussed above.

Effect of pCa on reduction of cytochrome *c* by 2-oxoglutarate

Table 1 presents the degree of reduction of cytochrome *c* by 0.5 mM-2-oxoglutarate as a function of pCa , during State-3 and State-4 respiration. It is seen that the dependence of reduction on pCa in State 4 is very similar to that presented for NAD(P) in Fig. 3, with half-maximal activation occurring at

Fig. 3. Dependence of the steady-state reduction of NAD(P)⁺ on the pCa of the medium during State-4 2-oxoglutarate oxidation

Reduction was measured in experiments of the type shown in Fig. 1(a), immediately before the addition of ADP. Curve A, the medium was that used for Fig. 1(a) and a range of $Ca^{2+}/EGTA$ buffers was used to give the pCa values shown. Values are means $\pm$ S.E.M. for four mitochondrial preparations from 6-month-old rats. Curve B, conditions were exactly as for curve A, but the mitochondrial preparations were from seven 24-month-old rats. Curve C, $MgCl_2$ was omitted from the medium. Curve D, P_i was omitted from the medium. In curves C and D the values are means, differing by no more than 10%, for two mitochondrial preparations from 6-month-old animals.

pCa 6.68. By contrast, there is no statistically significant variation in the degree of reduction of cytochrome *c* with pCa in State 3. Results obtained with NAD(P) are also included in Table 1, and here, also, pCa has no detectable effect on reduction in State 3. This apparent lack of sensitivity to Ca^{2+} presumably reflects activation of 2-oxoglutarate dehydrogenase by a decreased mitochondrial $[ATP]/[ADP]$ ratio consequent on the addition of ADP to the suspension, in keeping with the sensitivity to adenine nucleotides noted of the insect flight-muscle enzyme by Hansford (1972) and described for heart by McCormack & Denton (1979). In addition, mitochondrial 2-oxoglutarate content tends to be higher in State 3 than in State 4 (Hansford & Johnson, 1975), which will minimize sensitivity to Ca^{2+} in State 3, on the basis of the kinetics of the isolated 2-oxoglutarate dehydrogenase (McCormack & Denton, 1979). Some sensitivity to Ca^{2+} does, however, remain in State 3, as shown by Denton *et al.* (1980), who measured O_2 uptake with 2-oxoglutarate. When ΔE_h is calculated

Table 1. *Effect of pCa on the steady-state reduction of NAD(P) and cytochrome c by 2-oxoglutarate in States 3 and 4* The reduction of NAD(P) in State 4 was measured as described for Fig. 3 (curve A); the reduction in State 3 was measured after the addition of 3 μ mol of ADP. Reduced cytochrome *c* was measured by dual-wavelength spectrophotometry as described in the Experimental section.

pCa	State 3			State 4		
	Reduction of NAD(P) (%)	Reduction of cytochrome <i>c</i> (%)	ΔE_h (mV)	Reduction of NAD(P) (%)	Reduction of cytochrome <i>c</i> (%)	ΔE_h (mV)
6.25	11.3 $\pm$ 0.3 (4)	8.2 $\pm$ 1.2 (3)	620.5	70.9 $\pm$ 1.8 (4)	28.3 $\pm$ 2.3 (3)	620.2
6.37	11.5 $\pm$ 0.1 (4)	10.1 $\pm$ 1.6 (3)	614.9	66.6 $\pm$ 2.8 (4)	29.8 $\pm$ 0.5 (3)	615.9
6.54	12.5 $\pm$ 0.6 (3)	10.1 $\pm$ 1.6 (3)	616.0	46.3 $\pm$ 3.5 (4)	24.1 $\pm$ 2.8 (3)	612.7
6.64	12.1 $\pm$ 0.5 (4)	10.9 $\pm$ 1.5 (3)	613.9	31.8 $\pm$ 3.7 (4)	21.2 $\pm$ 2.8 (3)	609.1
6.75	13.0	8.4 $\pm$ 1.0 (3)	621.8	20.1 $\pm$ 2.5 (3)	13.1 $\pm$ 1.9 (3)	616.2
6.88	12.0	10.2 $\pm$ 1.1 (3)	615.2	9.6 $\pm$ 0.3 (3)	11.9 $\pm$ 2.3 (3)	608.0
EGTA	9.9 $\pm$ 0.8 (4)	6.9 $\pm$ 0.5 (3)	623.3	8.8 $\pm$ 0.9 (5)	6.8 $\pm$ 0.6 (3)	622.6

Table 2. *Effect of senescence on the rate of response of 2-oxoglutarate dehydrogenase activity to addition of Ca²⁺/EGTA buffers and EGTA to mitochondrial suspensions*

Oxidation of NAD(P)H was effected in (A) by the addition of 4 μ mol of EGTA and 20 μ mol of NaCl to mitochondria incubated as described for Fig. 1(b). In (B) conditions were the same, except that 10 mM-NaCl was present from the beginning of the incubation and MgCl₂ was present as indicated. In (C) the rate of reduction of NAD(P) was measured as in Fig. 1(a), but with a 10-fold higher chart speed; Mg²⁺ was present as indicated. Values given are the means $\pm$ S.E.M. for determinations representing different mitochondrial preparations (numbers in parentheses). No preparation used in series (A) was also used in (B). Statistical significance of differences is indicated (N.S., not significant).

		Rate of oxidation of NAD(P)H [% of total NAD(P)H/min]				<i>P</i>
	pCa	[Mg ²⁺] (mM)	6-month-old rats	24-month-old rats	Difference (%)	
(A)	6.64	1	105 $\pm$ 6 (5)	62.8 $\pm$ 7.1 (7)	-40	<0.005
(B)	6.64	—	75.7 $\pm$ 3.2 (4)	49.7 $\pm$ 6.0 (4)	-34	<0.01
	6.15	1	121 $\pm$ 7.0 (4)	77.6 $\pm$ 10.5 (4)	-36	<0.025
		Rate of reduction of NAD(P) ⁺ [% of total NAD(P) ⁺ /min]				
	pCa	[Mg ²⁺] (mM)				
(C)	6.64	—	262 $\pm$ 6 (4)	236 $\pm$ 18 (4)	-10	N.S.
	6.15	1	173 $\pm$ 6 (4)	173 $\pm$ 19 (4)	0	N.S.

for the span NADH–cytochrome *c* (Table 1), there is no statistically significant variation with pCa in State 3, but a significant increase caused by Ca²⁺ in State 4 (cf. ΔE_h at pCa 6.25 and 6.88, $P < 0.01$). The redox span when EGTA is used alone (Table 1) appears anomalous. This increase in ΔE_h will allow the generation of higher adenine nucleotide phosphorylation potentials in State 4 (see Erecińska *et al.*, 1974, 1978), and, if true for the physiologically important ADP-limited respiratory states between 3 and 4, the maintenance of high rates of oxidative phosphorylation without allowing the adenine nucleotide phosphorylation potential to fall to unacceptably low values (for a review, see Hansford, 1980). The quantitative arguments here would be improved by measuring the concentrations of free NAD⁺ and NADH; however, there is no satisfactorily active dehydrogenase for this to be done

from hydrogen donor and acceptor ratios, in heart mitochondria.

Impact of senescence on the sensitivity to pCa of NAD(P) reduction by 2-oxoglutarate

When the inactivation of 2-oxoglutarate dehydrogenase on adding EGTA to a mitochondrial suspension is monitored as shown in Fig. 1(b), a much lower rate is obtained when heart mitochondria from senescent rats (24 months old) are used, as opposed to young adults (6 months old). Results obtained with several mitochondrial preparations from each age are compiled in Table 2, which shows that the rate of inactivation is diminished by 40% in senescence (Table 2A). An experiment of similar design, but differing in that Na⁺ was present from the beginning of the experiment, gave a decrement

with senescence of a similar magnitude (Table 2B). It is noted that different sets of mitochondrial preparations were used in the two portions of the study, which involved nine young adult and eleven senescent animals in all. The effect of Mg²⁺ in enhancing EGTA-induced enzyme inactivation, which was alluded to above, is also apparent in Table 2. The rate of enzyme activation, as measured by the reduction of NAD(P), is by contrast not diminished with age (Table 2C).

The mechanism of the age-linked change in enzyme inactivation was presumed to lie at the level of Ca²⁺ egress from the mitochondria, and this was measured directly by using the Arsenazo III technique. It was found that the rate of Na⁺-induced Ca²⁺ egress in the presence of Ruthenium Red, to block re-uptake (see Crompton *et al.*, 1976b), was $6.76 \pm 0.21(7)$, $10.5 \pm 0.7(5)$ and $11.0 \pm 0.6(5)$ nmol/min per mg of protein at 25°C for mitochondria from young rats that had been loaded with 11, 33 and 42 nmol of Ca²⁺/mg respectively. The corresponding values for mitochondria from the old rats were $4.48 \pm 0.51(7)$, $7.34 \pm 0.51(7)$ and $8.26 \pm 0.56(6)$, indicating decrements with senescence of 34, 30 and 25% respectively, with *n* indicating the number of mitochondrial preparations and *P* < 0.01 in all cases. The endogenous Ca²⁺ content of approx. 2–3 nmol/mg was not measured in these experiments. In another experimental design, very low Ca²⁺ loads were applied and endogenous Ca²⁺ content was measured. Results indicated a 37% decrement with age, at a total Ca²⁺ content of 5 nmol/mg, rates being $4.87 \pm 0.44(4)$ nmol/min per mg for the 6-month-old animals and $3.09 \pm 0.38(4)$ for the 24-month-old animals. The preparations and animals used were different from those used above. Thus there is a decreased ability to transport Ca²⁺ out of heart mitochondria in old age, and this accords with the decreased rate of inactivation of 2-oxoglutarate dehydrogenase on adding EGTA. The effect is not due to a non-specific deterioration of mitochondrial structure or to an increased contamination with non-mitochondrial protein in the preparation from the old animals, as other activities are undiminished with age on a protein basis. Thus State-3 rates of oxidation of pyruvate plus malate, glutamate plus malate and 2-oxoglutarate (0.5 mM and 20 mM) plus malate were undiminished with age, as was the activity of 2-oxoglutarate dehydrogenase in Triton-extracted mitochondria, measured under *V*_{max} conditions (see the Experimental section). This was $123 \pm 10(4)$ nmol/min per mg at 25°C for the 6-month-old and $123 \pm 6(4)$ for the 24-month-old rat. Undiminished rates of oxidation of several non-lipid substrates have also been reported by Hansford (1978). The inference is that there is a specific decline with age in Ca²⁺/*n*Na⁺ exchange, with the decrement at 42 nmol of Ca²⁺/mg of

mitochondrial protein implying a changed *V*_{max}. An additional change in *K*_m is also possible, however. Direct studies of Ca²⁺ uptake also reveal decreases in activity in old age, which are statistically significant, though smaller than the changes in the egress pathway (results not shown); here, then, the agreement with the results of the enzyme-activation studies, which show no change with age (Table 2C), is not so good.

Fig. 3 presents the dependence on pCa of NAD(P) reduction in the steady state in preparations from senescent, as well as young adult, animals. There is a small shift in the curves, with 50% activation at pCa 6.58 and 6.64 in 6-month-old and 24-month-old rats respectively. More apparent is the significant (*P* < 0.05) difference at pCa 6.25; the region pCa 6.15–6.25 reflects the beginning of a phase of mitochondrial pathology seen when the Ca²⁺ load is too high, as shown by Hansford & Cohen (1978) and discussed by Denton *et al.* (1980) and Hansford (1981). The decreased tolerance to Ca²⁺ of the mitochondria from the old animals is consistent with a decrease in the activity of the Ca²⁺/*n*Na⁺-egress pathway (above) and a smaller decline in the electrogenic Ca²⁺-uptake process.

Dependence of 2-oxoglutarate dehydrogenase activity on mitochondrial Ca²⁺ content

Fig. 4 shows the relation between the reduction of mitochondrial NAD(P) by 0.5 mM-2-oxoglutarate and mitochondrial Ca²⁺ content, determined by atomic-absorption spectrophotometry. It is seen that the endogenous content of 3–4 nmol/mg of protein found on preparation is enough for maximal activation; 50% activation is achieved when this content is lowered to 1 nmol/mg, by partial withdrawal with small quantities of EGTA. Williamson & Murphy (1981) have determined the free Ca²⁺ concentration of the matrix of liver mitochondria to be in the region 60–150 μM, with loads of 10–36 nmol/mg, and these experiments have been taken to cast doubt (Williamson & Cooper, 1980) on the physiological importance of the activation by Ca²⁺ of NAD-linked isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase, an activation with *K*_{0.5} values for Ca²⁺ of about 1 μM (Denton *et al.*, 1978; McCormack & Denton, 1979). The present work suggests that this difficulty is more apparent that real, by emphasizing that modulation of 2-oxoglutarate dehydrogenase activity occurs at much lower total mitochondrial Ca²⁺ loads than those used by Williamson & Murphy (1981). If the activity coefficient of 0.0035 for intramitochondrial Ca²⁺ derived by Williamson & Murphy (1981) is applied to the data presented in Fig. 4, then 2-oxoglutarate dehydrogenase is seen to be responding to Ca²⁺ in the concentration range 2–6 μM,

Fig. 4. Dependence of 2-oxoglutarate dehydrogenase activity on the mitochondrial content of Ca^{2+}

Mitochondria (4 mg of protein) were added to 2 ml of medium containing 0.13 M-KCl, 20 mM-Hepes (potassium salt), 5 mM- P_i (potassium salt) and 0.25 mM ADP, pH 7.2. 2-Oxoglutarate (1 μmol) and L-malate (1 μmol) were added 10 min later, and the fluorescence of NAD(P) was monitored. At 12.5 min, a 1.5 ml portion was removed and the mitochondria were recovered by centrifugation and assayed for Ca^{2+} content by atomic-absorption spectrophotometry as described in the Experimental section. Each symbol characterizes a result from one particular mitochondrial preparation; four preparations were used in all. Mitochondrial Ca^{2+} contents below 2 nmol/mg were achieved by the addition of small amounts of EGTA + NaCl at the beginning of the experiment; the content of 14 nmol/mg was achieved by the addition of 30 nmol of CaCl_2 .

which is much nearer to the $K_{0.5}$ values determined with the isolated enzyme.

General discussion

We have exploited the reduction of mitochondrial NAD(P) by non-saturating concentrations of 2-oxoglutarate as a technique for studying the activation of 2-oxoglutarate dehydrogenase by Ca^{2+} when the enzyme is situated in its intramitochondrial locale. The disadvantage of the technique is that it responds to both NADH and NADPH. However, in coupled mitochondria, any change in fluorescence can be attributed very largely to a change in NADH content, as discussed above. The advantages are that it allows continuous measurement of activity, including rates of increase and decrease, and that it can be applied to ADP-limited states such as State 4, where high $[\text{NADH}]/[\text{NAD}^+]$ ratios make the effect of Ca^{2+} particularly pronounced, as shown for the purified enzyme by Lawlis & Roche (1980). This is not true of the

oxygen-electrode technique (Denton *et al.*, 1980), where the expression of dehydrogenase activity requires the addition of ADP. The pronounced effect of Ca^{2+} on the reduction of NAD(P) by 2-oxoglutarate is in clear contrast with results obtained with pyruvate as substrate (Hansford & Cohen, 1978; Hansford, 1981), where the percentage reduction of NAD(P) is unchanged with pCa in State 4, despite large effects of pCa on the content of enzymically active dephosphopyruvate dehydrogenase. Enhanced catalytic activity and a low flux enforced by the low activity of the respiratory chain are reconciled in the case of pyruvate dehydrogenase by a rise in the steady-state $[\text{acetyl-CoA}]/[\text{CoASH}]$ ratio, with a decrease in pCa (Hansford & Cohen, 1978). Conceivably the $[\text{succinyl-CoA}]/[\text{CoASH}]$ ratio rises less, and the $[\text{NADH}]/[\text{NAD}^+]$ ratio is more important, in the feedback inhibition of 2-oxoglutarate dehydrogenase.

The impact of senescence on energy metabolism is a major concern of this laboratory. The present work shows a modest shift in the dependence of NAD(P) reduction by 2-oxoglutarate on pCa with the age of the donor animal (Fig. 3). This is attributed to a greater decline in the activity of the mitochondrial $\text{Ca}^{2+}/n\text{Na}^+$ exchange than in the activity of the electrogenic entry pathway. Accordingly, the rate of activation of 2-oxoglutarate dehydrogenase by extramitochondrial Ca^{2+} is unimpaired, whereas the rate of inactivation on Ca^{2+} withdrawal is lowered by 40% in senescence (Table 2). There is no change with age in the Ca^{2+} -sensitivity of the 2-oxoglutarate dehydrogenase, as experiments with Triton-solubilized mitochondria (see the Experimental section) reveal 25% of V_{max} activity at $\text{pCa } 6.48 \pm 0.02(4)$ for the 6-month-old animals and at $\text{pCa } 6.49 \pm 0.05(4)$ for the 24-month-old animals, at 0.25 mM-2-oxoglutarate and pH 7.2. Recent work has shown that ATP, ADP and P_i concentrations vary with the heart cycle (Fossel & Ingwall, 1980). It is possible, though speculative, that mitochondrial Ca^{2+} transport parallels oscillations of cytosolic Ca^{2+} concentration during the heart cycle, and that this is somewhat damped in the aging heart. There is evidence for an altered handling of Ca^{2+} in aging myocardium, with a decreased responsiveness to catecholamines, which is overcome at elevated concentrations of Ca^{2+} in the perfusion fluid (see Lakatta, 1980) and a decreased activity in Ca^{2+} uptake of isolated sarcoplasmic reticulum (Froehlich *et al.*, 1978).

Finally, the finding that 2-oxoglutarate dehydrogenase responds to mitochondrial Ca^{2+} content in the range 0.5–1.5 nmol/mg (Fig. 4), together with the inference of similar sensitivity for the pyruvate dehydrogenase phosphatase (Hansford, 1981), strongly supports the idea (Denton *et al.*, 1978; Denton & McCormack, 1980) that intramitochondrial Ca^{2+}

concentration is a modulator of flux through pyruvate dehydrogenase and the tricarboxylate cycle under physiological conditions. The mitochondrial content of Ca²⁺ *in vivo* has been found by electron-probe analysis to be less than 1 nmol/mg dry wt. in smooth muscle (Somlyo *et al.*, 1979). It is suggested that an increased cytosolic Ca²⁺ concentration, generated during the state of high work-load, activates pyruvate dehydrogenase, NAD-linked isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase via enhanced mitochondrial Ca²⁺ uptake and an increased matrix Ca²⁺ concentration. At the same time, the mitochondrial glycerol 3-phosphate dehydrogenase, important in the oxidation of glycolytic reducing power in some muscles (for a review see Sacktor, 1975), is also activated by Ca²⁺ in the range pCa 6–8 (Hansford & Chappell, 1967), but faces the cytosol, rather than the mitochondrial matrix compartment (Klingenberg & Buchholz, 1970), and thus probably senses the pCa of the cytosol directly.

We thank Dr. Bruce Baum for the atomic-absorption spectrophotometry.

References

- Chance, B. & Williams, G. R. (1956) *Adv. Enzymol. Relat. Subj. Biochem.* **17**, 65–134
- Chappell, J. B. (1968) *Br. Med. Bull.* **24**, 150–157
- Crompton, M. & Heid, I. (1978) *Eur. J. Biochem.* **91**, 599–608
- Crompton, M., Sigel, E., Salzmann, M. & Carafoli, E. (1976a) *Eur. J. Biochem.* **69**, 429–434
- Crompton, M., Capano, M. & Carafoli, E. (1976b) *Eur. J. Biochem.* **69**, 453–462
- DeHaan, E. J. & Tager, J. M. (1968) *Biochim. Biophys. Acta* **153**, 98–112
- Denton, R. M. & McCormack, J. G. (1980) *FEBS Lett.* **119**, 1–8
- Denton, R. M., Richards, D. A. & Chin, J. G. (1978) *Biochem. J.* **176**, 899–906
- Denton, R. M., McCormack, J. G. & Edgell, N. J. (1980) *Biochem. J.* **190**, 107–117
- Erecińska, M., Veech, R. L. & Wilson, D. F. (1974) *Arch. Biochem. Biophys.* **160**, 412–424
- Erecińska, M., Wilson, D. F. & Nishiki, K. (1978) *Am. J. Physiol.* **234**, C82–C89
- Fossel, E. T. & Ingwall, J. S. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 2113 (Abstr. 2683)
- Froehlich, J. P., Lakatta, E. G., Beard, E., Spurgeon, H. A., Weisfeldt, M. L. & Gerstenblith, G. (1978) *J. Mol. Cell. Cardiol.* **10**, 427–438
- Hansford, R. G. (1972) *FEBS Lett.* **21**, 139–141
- Hansford, R. G. (1978) *Biochem. J.* **170**, 285–295
- Hansford, R. G. (1980) *Curr. Top. Bioenerg.* **10**, 217–278
- Hansford, R. G. (1981) *Biochem. J.* **194**, 721–732
- Hansford, R. G. & Chappell, J. B. (1967) *Biochem. Biophys. Res. Commun.* **27**, 686–692
- Hansford, R. G. & Cohen, L. (1978) *Arch. Biochem. Biophys.* **191**, 65–81
- Hansford, R. G. & Johnson, R. N. (1975) *J. Biol. Chem.* **250**, 8361–8375
- Johnson, R. N. & Hansford, R. G. (1977) *Biochem. J.* **164**, 305–322
- Klingenberg, M. & Buchholz, M. (1970) *Eur. J. Biochem.* **13**, 247–252
- Lakatta, E. G. (1980) in *The Aging Heart* (Weisfeldt, M. L., ed.), pp. 77–100, Raven Press, New York
- Lawlis, V. B. & Roche, T. E. (1980) *Mol. Cell. Biochem.* **32**, 147–152
- Lee, C. O. & Fozzard, H. A. (1975) *J. Gen. Physiol.* **65**, 595–608
- McCormack, J. G. & Denton, R. M. (1979) *Biochem. J.* **180**, 533–544
- Mitchell, P. (1966) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Bodmin
- Murphy, J. B. & Kies, M. W. (1960) *Biochim. Biophys. Acta* **45**, 382–384
- Portzehl, H., Caldwell, P. C. & Rüegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591
- Sacktor, B. (1975) *Adv. Insect Physiol.* **7**, 267–347
- Scarpa, A. (1979) *Methods Enzymol.* **56**, 301–338
- Somlyo, A. P., Somlyo, A. V. & Shuman, H. (1979) *J. Cell Biol.* **81**, 316–335
- Veloso, D., Guynn, R. W., Oskarsson, M. & Veech, R. L. (1973) *J. Biol. Chem.* **248**, 4811–4819
- Williamson, J. R. & Cooper, R. H. (1980) *FEBS Lett.* **117**, K73–K85
- Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–513
- Williamson, J. R. & Murphy, E. (1981) *Adv. Exp. Med. Biol.* in the press