Biochem. J. (1990) 268, 421–428 (Printed in Great Britain)

Influence of NAD-linked dehydrogenase activity on flux through oxidative phosphorylation

Rafael MORENO-SÁNCHEZ,*† Barbara A. HOGUE* and Richard G. HANSFORD*
*Energy Metabolism and Bioenergetics Section, Laboratory of Cardiovascular Science, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, U.S.A., and †Departamento de Bioquimica, Instituto Nacional de Cardiologia, Mexico, D.F. 14080, Mexico

1. We have examined systematically the relationship between the percentage reduction of cardiac mitochondrial NAD and the flux through oxidative phosphorylation, as measured by O_2 uptake. Reduction of NAD was varied by varying the concentration of palmitoyl-L-carnitine, pyruvate, 2-oxoglutarate or glutamate in the presence of malate as the oxidizable substrate. 2. In the presence of ADP (State 3 respiration) there was a substantially linear positive relationship between O_2 uptake and the percentage reduction of NAD. Coupled respiration in the absence of ADP also showed an increase with increasing NADH, with the exact shape of the relationship being variable. 3. When pyruvate and 2-oxoglutarate dehydrogenase activity were increased by increasing medium Ca^{2+} concentration within the range 5 nM to 1.23 μM, at non-saturating substrate concentrations, there was again a positive relationship between O_2 uptake and the reduction of NAD; however, rates of O_2 uptake tended to be higher at given values of NAD reduction when the incubation medium contained Ca^{2+}. This is taken to indicate an activation by Ca^{2+} of the enzymes of phosphorylation or of the respiratory chain, in addition to the dehydrogenase activation. 4. When carboxyatractyloside plus ADP were used to generate 50 % State 3 rates of O_2 uptake with pyruvate or 2-oxoglutarate, sensitivity to Ca^{2+} was retained. However, when oligomycin plus 1 mM-ADP and 1 mM-ATP were used to generate 50 % State 3, no such dependence was seen. 5. The results are interpreted to indicate a substantial role for substrate dehydrogenation in the overall regulation of oxidative phosphorylation when substrates are available at near-physiological concentrations.

INTRODUCTION

It has been shown by a number of investigators that the control of oxidative phosphorylation by isolated mitochondria is multifaceted in nature (Groen *et al.*, 1982; Bohnensack *et al.*, 1982; Doussiere *et al.*, 1984; Baggetto *et al.*, 1984; Moreno-Sánchez, 1985*b*). Application of the theory of metabolic control developed by Kacser & Burns (1973) and Heinrich & Rapoport (1974) allowed the identification of the ATP/ADP carrier, the ATP synthase, the cytochrome oxidase, the cytochrome *b–c* complex and the dicarboxylate carrier as important sites at which control is exerted (Groen *et al.*, 1982; Bohnensack *et al.*, 1982; Doussiere *et al.*, 1984; Baggetto *et al.*, 1984; Moreno-Sánchez, 1985*b*). However, these studies were performed with mitochondria incubated in the presence both of saturating concentrations of succinate (5–20 mM) and of the site I inhibitor of electron transport, rotenone: under these conditions, the contribution of NAD-linked dehydrogenases to metabolic control is clearly excluded. Those reports involving NAD-linked substrates (Doussiere *et al.*, 1984; Davis & Davis-Van Thienen, 1984) used saturating concentrations (> 1 mM), again minimizing a control function for substrate permeation or dehydrogenation.

By contrast, the tissue concentrations of pyruvate and 2-oxoglutarate in the heart are below 1 mM (Williamson & Corkey, 1969; Bünger & Permanetter, 1984; Weisner *et al.*, 1988) and the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase reactions are clearly far from equilibrium, implying finite control strength. Moreover, there is recent evidence from ^{31}P-n.m.r. studies that increased workloads in heart are associated with a lack of change in the availability of free ADP (Kantor *et al.*, 1986; Balaban *et al.*, 1986; From *et al.*, 1986; Katz *et al.*, 1987, 1988, 1989) but may be associated with an increased reduction of mitochondrial NAD (Katz *et al.*, 1987). This would be consistent with an activation of mitochondrial dehydrogenases, possibly by Ca^{2+} (see Denton & McCormack, 1985; Hansford, 1985, and references therein) and could lead to increased flux through oxidative phosphorylation in the absence of a change in adenine nucleotide phosphate potential (Hansford, 1980). When dehydrogenase activation by increased cardiac workload is curtailed, as when the heart is perfused throughout with high concentrations of pyruvate or is treated with Ruthenium Red, then a correlation between rates of O_2 uptake and cytosolic free ADP concentration is evident (From *et al.*, 1986; Katz *et al.*, 1988). An effect of dehydrogenase activation is also clearly discernible in hepatocytes, where the Ca^{2+}-mobilizing hormones give rise to an increased O_2 uptake at an unchanged adenine nucleotide phosphate potential (Balaban & Blum, 1982; Charest *et al.*, 1983; Assimacopoulos-Jeannet *et al.*, 1983), the mechanism apparently being an activation of pyruvate and 2-oxoglutarate dehydrogenases by Ca^{2+} (Sugden *et al.*, 1980; Staddon & Hansford, 1986) and a consequent elevation of the mitochondrial $NADH/NAD^+$ ratio (Balaban & Blum, 1982; Charest *et al.*, 1983; Staddon & Hansford, 1987).

In the present work we have sought to investigate the control of oxidative phosphorylation under conditions which do not exclude dehydrogenase-level control. Our approach has been to examine in a more quantitative way the relationship between the percentage reduction of mitochondrial NAD and the rate of O_2 uptake, in the presence and absence of ADP, when reduction of NAD is varied by varying the concentration of oxidizable substrate or, in the case of oxidations involving flux through pyruvate and 2-oxoglutarate dehydrogenases, the concentration of ionized Ca^{2+}. The study differs from that of Koretsky & Balaban (1987) in that respiratory activity is presented as a function of concentration for each substrate separately and in its emphasis on the activation of dehydrogenases by Ca^{2+}.

A preliminary report of this work was given at the 73rd Annual Meeting of the Federation of American Society for Experimental Biology in New Orleans, Louisiana (Moreno-Sánchez *et al.*, 1989).

Abbreviation used: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

MATERIALS AND METHODS

Preparation and incubation of mitochondria

Rat heart mitochondria were isolated as described previously (Moreno-Sánchez & Hansford, 1988). Mitochondria were incubated in a standard medium, comprising 0.12 M-KCl, 25 mM-K-Mops, 0.5 mM-EGTA, 5 mM-potassium phosphate, 10 mM-NaCl, pH 7.2 at 25 °C and with the additions noted in the appropriate Figure legends. Ca^{2+}/EGTA buffers were made up and used as described previously (Moreno-Sánchez & Hansford, 1988). O_2 uptake was measured by means of a Clark-type O_2-electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). The solubility of O_2 in equilibrium with air was taken to be 508 nmol/ml at 25 °C.

Determination of the percentage reduction of mitochondrial nicotinamide nucleotides

The percentage reduction of mitochondrial NAD was determined by differential absorbance measurement of the mitochondrial suspensions at 340–370 nm, using an Aminco DW2c spectrophotometer. An atmosphere of 100% O_2 was maintained for these studies. Differential absorbance values achieved with the various substrate combinations were calibrated by reference to minimal and maximal values for the mitochondrial suspension. The minimum [0% NAD(P)H] was achieved by adding 1–2 mM-ADP to a suspension incubated for 5–6 min in the absence of oxidizable substrate; 1 μM-FCCP, added instead of ADP, gave identical values after correction for quenching by FCCP itself. Maximal reduction [100% NAD(P)H] was generated by adding consecutively rotenone, malate and glutamate; this was done at the end of each experiment (see Fig. 1).

The efficacy of this calibration protocol was checked by extraction of nucleotides and subsequent enzymic determination. Mitochondria were extracted either with $HClO_4$, for determination of NAD^+ and $NADP^+$, or with KOH in 50% (v/v) ethanol, for determination of NADH and NADPH. The procedure was as previously described (Williamson & Corkey, 1969). NAD^+ and $NADP^+$ were determined after neutralization of the acidic extract with a mixture of tri-n-octylamine/1,1,2-trichlorotrifluoroethane (1:1, v/v) (Khym, 1975) using glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. This enzyme reacts with both NAD^+ and $NADP^+$, but has more affinity for $NADP^+$ (Kurlansky *et al.*, 1988). In the presence of 0.5 mM-glucose 6-phosphate, the reaction with $NADP^+$ was complete within 30 s at 30 °C when using 0.3 units of this enzyme/ml; reaction with NAD^+ required up to 10 min for completion. NADH and NADPH were determined as described elsewhere (Williamson & Corkey, 1969).

Presentation of results

The values given in this paper represent means ± S.D. obtained from the number of mitochondrial preparations indicated in parentheses. An exception to this is the use of S.E.M. for the error bars in Figs. 2, 4, 6, 7 and 8.

Materials

Nagarse was obtained from Enzyme Development Corp. (New York, NY, U.S.A.); EGTA and carboxyatractyloside were from Sigma Chemical Co.; oligomycin was from Boehringer Mannheim.

RESULTS AND DISCUSSION

Method of determination of NADH

We chose to use differential absorbance measurements of mitochondrial suspensions as an index of the degree of reduction of mitochondrial NAD because absorbance, unlike fluorescence, is insensitive to the environment of NAD(P)H (Vinogradov *et al.*, 1972), and to temperature. Reference of intermediate degrees of reduction of NAD(P) to complete oxidation and complete reduction (see the Materials and methods section) was validated by nucleotide extraction and enzymic assay (Table 1). Oxidation of NAD(P)H induced by ADP was in good agreement with protocols using FCCP or valinomycin to generate maximal oxidation of nicotinamide nucleotides. Clearly, the majority of the absorbance signal is attributable to NADH. Earlier work (La Noue *et al.*, 1970; Hansford & Johnson, 1975) has shown that the NADP of heart mitochondria remains highly reduced unless the mitochondria are uncoupled, and thus substrate-dependent differences in absorbance in the present study can be assumed to reflect differences in the degree of reduction of NAD.

Effect of different oxidizable substrates

***(a)* Palmitoyl-L-carnitine.** Fig. 1 presents results of experiments in which the steady state reduction of mitochondrial NAD was measured with palmitoyl-L-carnitine as substrate, in both State 3 and State 4 (see Chance & Williams, 1955, for terminology). It also gives an example of the calibration protocol, as described in the Materials and methods section. It is noted that this allows the determination of 0% reduction of NAD, as well as the value for 100% reduction which was routinely obtained at the end of each experiment.

Table 1. Enzymic determination of nicotinamide nucleotides

Heart mitochondria (1.3–1.8 mg of protein/ml) were incubated in the standard medium described in the Materials and methods section in the absence of oxidizable substrates for 5 min. Then 2 mM-ADP, 2 μM-FCCP or 3 μM-valinomycin (valino) was added, as indicated. The reaction was stopped 4 or 5 min later with 3% (v/v) $HClO_4$ or ethanolic (50%, v/v) KOH for the estimation of oxidized and reduced nucleotides respectively. Details are given in the Materials and methods section. In another series of experiments, the reaction was continued by addition of 2.5 μM-rotenone (Rot), 5 mM-L-malate (Mal) and 5 mM-L-glutamate (Glu). The reaction was quenched, as described above, 2–3 min later.

	Nucleotide (nmol/mg of protein)			
Experimental condition	NAD^+	$NADP^+$	NADH	NADPH
1. No substrates + ADP	5.2 ± 1.2 (5)	1.35 ± 0.17 (5)	0.3 ± 0.2 (4)	0.2 (2)
2. No substrates + FCCP	6.0 ± 1.5 (3)	1.57 ± 0.28 (3)	0.4 (2)	0.2 (2)
3. No substrates + valino	6.1 ± 2.0 (3)	1.01 ± 0.10 (3)	0.4	0
4. Expt. 1 + Rot + Mal + Glu	0.6 ± 0.1 (5)	0.07 ± 0.04 (5)	4.4 ± 0.3 (4)	0.5 (2)
5. Expt. 2 + Rot + Mal + Glu	0.9 ± 0.4 (3)	0.08 ± 0.06 (3)	5.2 (2)	0.7 (2)
6. Expt. 3 + Rot + Mal + Glu	0.6 ± 0.2 (3)	0.08 ± 0.06 (3)	4.7	0.5

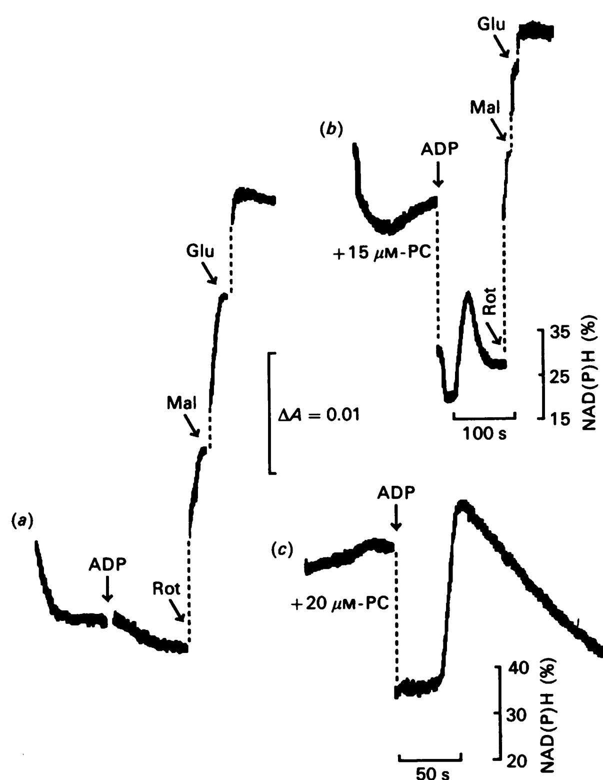

Fig. 1. Calibration of the absorbance signal and effect of palmitoyl-L-carnitine concentration on mitochondrial redox state

(a) Heart mitochondria (1.3 mg/ml) were incubated in 2 ml of an O_2-saturated standard medium at 25 °C with no added oxidizable substrates, and the absorbance difference at 340–370 nm was measured. After 5 min of incubation, 2 mM-ADP was added. Other additions were 2.5 μM-rotenone (Rot), 5 mM-malate (Mal) and 5 mM-glutamate (Glu). (b), (c) Mitochondria (1.3 mg/ml) were incubated with two different concentrations of palmitoyl-L-carnitine (PC)+1 mM-malate. ADP (1 mM) was added after 5 min of incubation. See the Materials and methods section for details of the determination of NAD(P)H as a percentage of total NAD(P).

Fig. 2 presents the quantitative relationship between the percentage reduction of NAD(P) and the rate of respiration at several concentrations of palmitoyl-L-carnitine; the redox data were derived from experiments of the type shown in Fig. 1. There was an essentially linear relationship between State 3 O_2 uptake and percentage of NAD(P)H when the palmitoyl-L-carnitine concentration was varied over the range 5–20 μM. This indicates a substantial role for substrate dehydrogenation in limiting flux through oxidative phosphorylation under these conditions of non-limiting ADP (1 mM) and phosphate (5 mM) availability.

For non-stimulated palmitoyl-L-carnitine oxidation, the percentage of NAD(P)H obtained prior to ADP addition was used in the plot (pseudo State 4), as values were not stable following ADP phosphorylation (Fig. 1). The shape of the relationship between percent NAD(P)H and O_2 uptake is unclear (Fig. 2), at least in part because of the uncoupling effect of the higher concentrations (30, 50 μM) of palmitoyl-L-carnitine. This was shown by a lower ADP/O ratio at 30 μM-palmitoyl-L-carnitine (2.90 ± 0.05, $n = 5$) than at 20 μM-palmitoyl-L-carnitine (3.1 ± 0.1, $n = 5$; $P < 0.05$).

A slight, but statistically significant ($P < 0.05$), effect of Ca^{2+} was observed on State 3 respiration and percentage reduction of NAD(P) when mitochondria were oxidizing 15 μM-palmitoyl-L-carnitine (Fig. 2). This may relate to activation by Ca^{2+} of

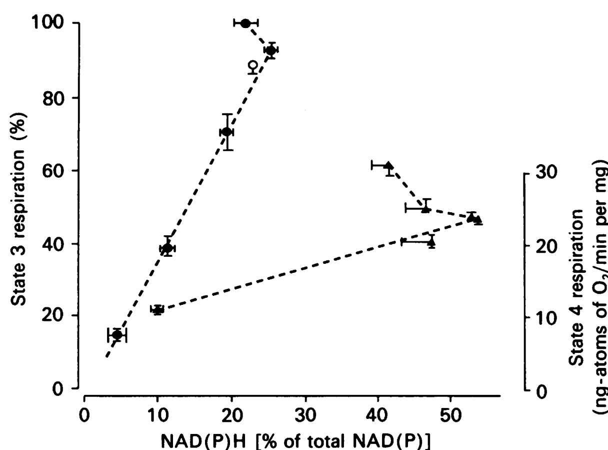

Fig. 2. Relationship between NAD(P)H and State 3 respiration in mitochondria oxidizing palmitoyl-L-carnitine

Different steady-state rates of respiration stimulated by 1 mM-ADP (State 3) were established after a prior 5 min incubation of mitochondria (1–1.4 mg/ml) with various concentrations of palmitoyl-L-carnitine (plus 1 mM-malate): i.e. 5, 10, 15, 20, 30 and 50 μM. In parallel experiments with the same mitochondrial preparation, the level of NAD(P)H was determined by measuring the absorbance difference at 340–370 nm (see Fig. 1a). The rate of pseudo State 4 respiration (in the absence of ADP; ▲) is presented in absolute units; the rate of State 3 respiration (●) was normalized to the rate obtained with 30 μM-palmitoyl-L-carnitine (317 ± 14 ng-atoms of O_2/min, $n = 5$). Also shown are results with 15 μM-palmitoyl-L-carnitine+1 mM-malate in the presence of 0.4 μM free Ca^{2+} (○). For clarity, the results obtained with 50 μM-palmitoyl-L-carnitine in State 3 were omitted: these were 20.5 ± 1.6% of NAD(P)H and 96 ± 1% (S.E.M.) of State 3 respiration, with 30 μM-palmitoyl-L-carnitine. Values of both % NAD(P)H and rate of State 4 respiration are significantly different at 5 and 20 μM-palmitoyl-L-carnitine ($P < 0.001$). The plot shows results obtained from five different mitochondrial preparations.

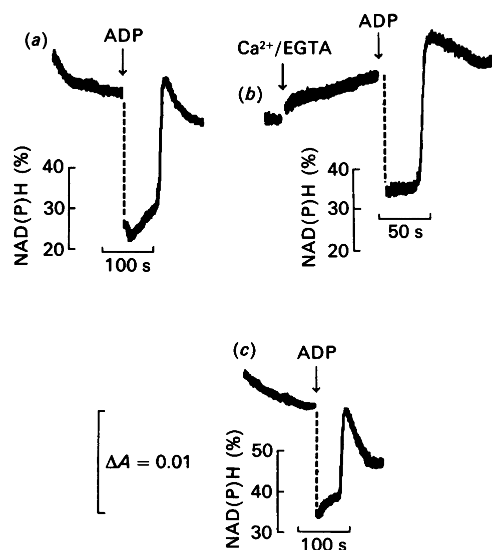

Fig. 3. Effect of pyruvate concentration and Ca^{2+} availability on mitochondrial redox state

Mitochondria (1.6 mg of protein/ml) were incubated with 0.5 mM-pyruvate (a,b) or 5 mM-pyruvate (c) in 2 ml of standard medium. In (b), a Ca^{2+}/EGTA buffer was added 2 min after the mitochondria to stabilize 0.4 μM free Ca^{2+}. ADP (1 mM) was added 5 min after the mitochondria and the steady-state level of NAD(P)H was determined as described in the legend to Fig. 1.

NAD–isocitrate dehydrogenase (Denton *et al.*, 1978) or 2-oxoglutarate dehydrogenase (McCormack & Denton, 1979), as tricarboxylate cycle activity is required for O_2 uptake with fatty acids in heart.

***(b)* Pyruvate.** Increasing concentrations of pyruvate resulted in increasing rates of respiration and percentage reduction of NAD(P), until saturation was achieved (Figs. 3 and 4). It is noteworthy that when various concentrations of Ca^{2+} were added to mitochondria oxidizing 0.5 mM-pyruvate in State 3, rates of O_2 uptake were obtained which exceeded that achieved with saturating pyruvate concentrations (5 and 7.5 mM), but in the absence of Ca^{2+}. The latter is referred to as 100% in Fig. 4. This suggests that Ca^{2+} is activating at sites other than the pyruvate dehydrogenase complex, as well as at this enzyme.

The effect of Ca^{2+} addition in increasing the percentage reduction of NAD(P) in State 4 (Fig. 3*b*), which appears as the displacement towards more reduced values in Fig. 4, clearly indicates activation of substrate dehydrogenation by Ca^{2+}. It is emphasized that the effect is quite distinct from the stimulation of O_2 uptake which is seen when mitochondria are exposed to massive loads of Ca^{2+} and which involves diminution of the mitochondrial protonmotive force and a tendency towards more oxidized nicotinamide nucleotide. A potential complication in studies of oxidative phosphorylation involving Ca^{2+} is that changes in membrane potential ($\Delta\psi$) caused, for instance, by the addition of ADP might be expected to affect the transmembrane Ca^{2+} ion gradient, owing to the electrophoretic nature of Ca^{2+} uptake (see Crompton, 1982; Hansford, 1985 for reviews). In practice, this seems to be a very minor effect under the conditions used in this paper, as direct studies of the mitochondrial Ca^{2+} ion gradient using the fluorescent chelating agents indo 1 and fura 2 trapped within the matrix show negligible changes on initiation of State 3 respiration (Moreno-Sánchez & Hansford, 1988; McCormack *et al.*, 1989; Wan *et al.*, 1989).

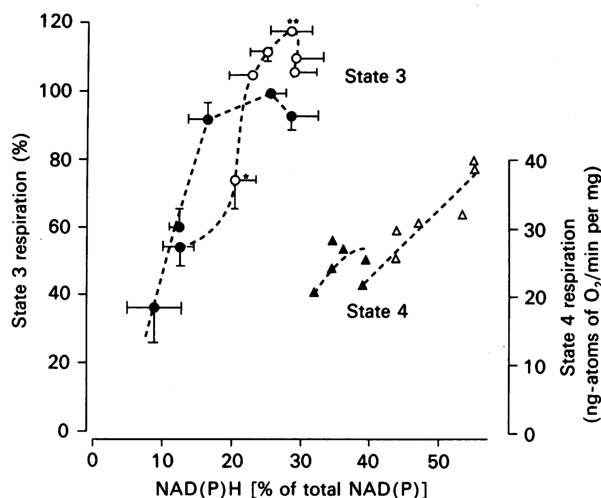

Fig. 4. Relationship between NAD(P)H and State 3 respiration in mitochondria oxidizing pyruvate

Different steady-state rates of respiration were established by incubating mitochondria (1–1.6 mg of protein/ml) with different pyruvate concentrations (+1 mM-malate), i.e. 0.25, 0.5, 1, 2, 5 and 7.5 mM (●, ▲). In parallel experiments with the same mitochondrial preparation the level of NAD(P)H was determined as described in the legend to Fig. 1. In another set of experiments the rate of respiration and the level of NAD(P)H were varied by adding different free Ca^{2+} concentrations in the presence of 0.5 mM-pyruvate+1 mM-malate (○, △): i.e. 5 nM (initial value), 66, 130, 225, 400, 800 and 1230 nM-Ca^{2+}: the Ca^{2+}/EGTA buffers were added 2 min after the mitochondria. The rate of State 3 respiration, as induced by the addition of 1 mM-ADP 5 min after the mitochondria, was normalized to the rate obtained with 5 mM-pyruvate+1 mM-malate (222 ± 17 ng-atoms of O_2/min per mg, mean ± S.E.M., $n = 8$). * Values of both % NAD(P)H and % State 3 respiration are significantly different at 5 and 66 nM-Ca^{2+} ($P < 0.05$). ** Values of % State 3 respiration are significantly different at 66 and 400 nM-Ca^{2+} ($P < 0.005$); no significant difference was found ($P < 0.1$) for values of % NAD(P)H. For clarity, the values of State 4 respiration only show the statistical means.

***(c)* 2-Oxoglutarate.** 2-Oxoglutarate dehydrogenase is probably largely flux-controlling for the segment of the tricarboxylate cycle from 2-oxoglutarate to malate (see Williamson, 1979, for review) and has been shown to be activated by Ca^{2+}. For these reasons, we investigated the effects of variation of 2-oxoglutarate concentrations and of Ca^{2+} upon steady-state levels of NAD(P) reduction (Fig. 5) and upon O_2-uptake (Fig. 6). The activation of 2-oxoglutarate dehydrogenase by Ca^{2+} is manifest as an increase in the level of NAD(P)H prior to addition of ADP (Fig. 5*b*). This reproduces prior work by Hansford & Castro (1981).

Increasing the concentration of 2-oxoglutarate from 0.25 mM to 10 mM induced increased rates of O_2 uptake and increased levels of NAD(P)H (Fig. 6). This shows that substantial flux limitation resides at the level of substrate dehydrogenation under these conditions (non-limiting P_i and ADP). Increased availability of free Ca^{2+} over the range 5 nM to 1.5 μM resulted in statistically significant increases in State 3 O_2 uptake when the substrate was 1 mM-2-oxoglutarate; however, there was a range (5–230 mM-Ca^{2+}) over which increases in NAD(P)H levels were undetectable (Fig. 6). We attribute this to a balanced activation by Ca^{2+} of the respiratory chain and/or phosphorylation system (ATP/ADP carrier plus ATP synthase). Activation by 0.4 μM-Ca^{2+} of the State 3 oxidation of 10 mM-2-oxoglutarate (125 ± 7% of control, $n = 7$, $P < 0.01$) supports this conclusion. Such an activation of the respiratory chain has been described by Halestrap (1989) and of the ATP/ADP carrier plus ATP synthase by Moreno-Sánchez (1985*a*,*b*). The failure of the Ca^{2+}-stimulated State 3 respiration to achieve 100% of the rate determined with 10 mM-2-oxoglutarate may reflect significant flux limitation by

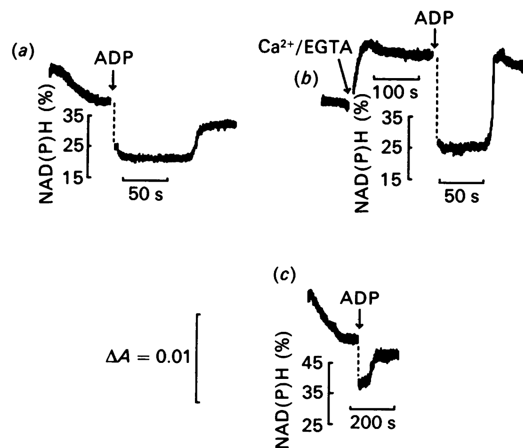

Fig. 5. Effect of 2-oxoglutarate concentration and Ca^{2+} availability on mitochondrial redox state

In (*a*) and (*c*) mitochondria (1.2 mg of protein/ml) were incubated with 1 mM (*a*) or 10 mM (*c*) 2-oxoglutarate. In (*b*), a Ca^{2+}/EGTA buffer was added to give 225 nM free Ca^{2+} ([2-oxoglutarate] = 1 mM). Other experimental details were as described in the legend of Fig. 3.

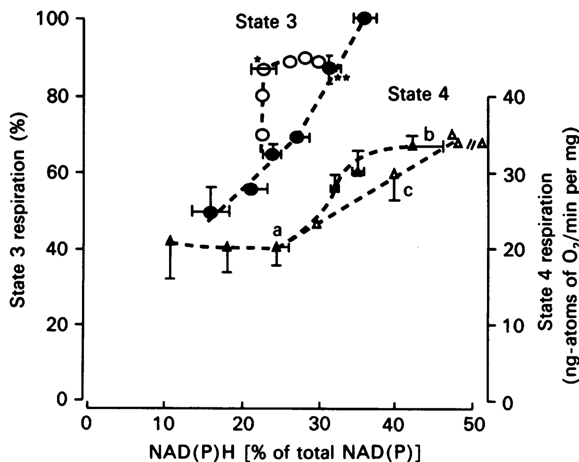

Fig. 6. Relationship between NAD(P)H and State 3 respiration in mitochondria oxidizing 2-oxoglutarate

Different steady-state rates of respiration were established after incubation of mitochondria (1–1.6 mg of protein/ml) with various concentrations of 2-oxoglutarate (●, ▲), i.e. 0.25, 0.5, 1, 2, 5 and 10 mM. Also shown (○, △) are results with mitochondria incubated with 1 mM-2-oxoglutarate and different free Ca^{2+} concentrations (see legend of Fig. 4 for values). The rate of State 3 respiration was normalized to the rate obtained with 10 mM-2-oxoglutarate (255 ± 16 ng-atoms of O_2/min per mg, mean ± S.E.M., $n = 8$). * Values of % State 3 respiration were significantly different at 5 and 225 nM-Ca^{2+} ($P < 0.001$); no significant difference was found for values of % NAD(P)H. ** Values of % NAD(P)H were significantly different at 1 mM-2-oxoglutarate + 225 nM-Ca^{2+} and 5 mM-2-oxoglutarate + 5 nM-Ca^{2+} ($P < 0.001$). a, $P < 0.01$ between values of % NAD(P)H at 0.25 and 1 mM-oxoglutarate: no significant difference was found for values of rate of State 4 respiration; b, $P < 0.001$ between values of % NAD(P)H, and rates of State 4 respiration at 1 and 10 mM-oxoglutarate; c, $P < 0.001$ between values of % NAD(P)H and $P < 0.05$ for values of State 4 respiration at 5 and 130 nM-Ca^{2+}.

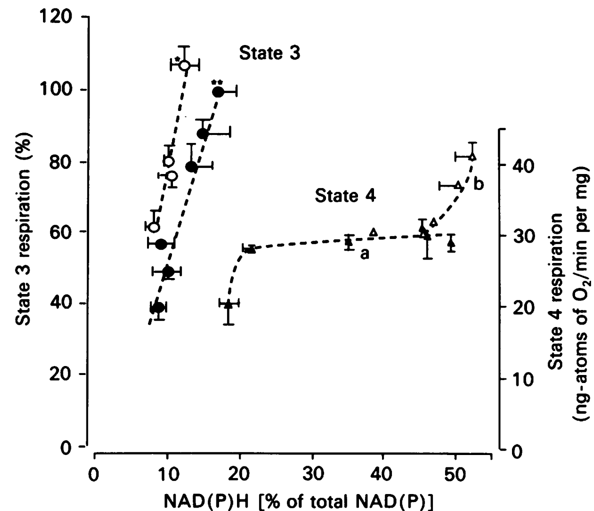

Fig. 7. Relationship between NAD(P)H and State 3 respiration in mitochondria oxidizing glutamate

Different rates of respiration were established after incubation of mitochondria (1–1.5 mg of protein/ml) with various concentrations of glutamate + 5 mM-malate (●, ▲), i.e. 0.25, 0.5, 1, 2, 5 and 10 mM. The level of NAD(P)H was determined as described in the legend to Fig. 1. Also shown (○, △) are results with 1 mM-glutamate + 5 mM-malate and different free Ca^{2+} concentrations, i.e. 5 nM (initial value), 66, 130, 225 and 400 nM. The rate of State 3 respiration was normalized to the rate obtained with 5 mM-glutamate + 5 mM-malate (195 ± 20 ng-atoms of O_2/min per mg, means ± S.E.M., $n = 6$). * Values of State 3 respiration were significantly different at 5 and 400 nM-Ca^{2+} ($P < 0.001$); no significant difference was found for values of % NAD(P)H. ** Values of % NAD(P)H with 1 mM-glutamate + 400 nM-Ca^{2+} were significantly different from those with 10 mM-glutamate + 5 nM-Ca^{2+} ($P < 0.05$); no significant difference was found for values of % State 3 respiration. a, Values of State 4 respiration and of % NAD(P)H were significantly different at 0.25 mM and 1 mM glutamate ($P < 0.05$ and $P < 0.001$ respectively); b, values of State 4 respiration and of % NAD(P)H were significantly different at 5 and 400 nM-Ca^{2+} ($P < 0.01$).

substrate permeation, rather than dehydrogenase activity, when the substrate is 1 mM-2-oxoglutarate.

The enzymic determination of NAD^+ and $NADP^+$ showed good agreement with the differential absorbance data of Fig. 6. The contents of NAD^+ and $NADP^+$ in State 3 conditions with 0.25, 1 and 10 mM-2-oxoglutarate were 5.4 ± 0.2 and 0.7 ± 0.2, 4.7 ± 0.2 and 0.6 ± 0.2, and 4.0 ± 0.2 and 0.6 ± 0.3 nmol/mg of protein respectively. For State 3 oxidation of 1 mM-2-oxoglutarate at 130 and 225 nM-Ca^{2+}, the results were 5 ± 0.5 and 0.6 ± 0.1, and 4.9 ± 0.7 and 0.5 ± 0.2 respectively (means ± S.D., $n = 3$ mitochondrial preparations).

The displacement of the curve relating State 3 O_2 uptake to the percentage reduction of NAD(P) to the left in the presence of Ca^{2+} is also seen when the mitochondria are provided with 1 mM-glutamate plus 1 mM-malate as substrate (Fig. 7). Again, this presumably involves a matching activation of the respiratory chain and/or the phosphorylation system by Ca^{2+}. A sensitivity to Ca^{2+} of glutamate oxidation in pseudo State 4 conditions (absence of adenine nucleotides) has been noted before (Hansford, 1985) and attributed to some flux control by 2-oxoglutarate dehydrogenase.

Significance of dehydrogenase activity at State 3 rates of 50% of maximum

To investigate the role of dehydrogenases in flux limitation under conditions where phosphorylation is less than fully active, we studied mitochondria treated with either sufficient carboxyatractyloside or oligomycin to diminish State 3 rates of O_2 uptake to 50% of the uninhibited value. When carboxyatractyloside was used as an inhibitor of the adenine nucleotide translocase (see Klingenberg, 1976), addition of Ca^{2+} to mitochondria oxidizing either 0.5 mM-pyruvate or 1 mM-2-oxoglutarate resulted in an increased rate of O_2 uptake, accompanied by an increased percentage reduction of NAD(P) (Fig. 8). Addition of ADP to these carboxyatractyloside-treated mitochondria resulted in an oxidation of NAD(P)H (Figs. 9*a* and 9*b*).

On the other hand, addition of Ca^{2+} to oligomycin-treated mitochondria oxidizing 0.5 mM-pyruvate or 1 mM-2-oxoglutarate gave no increase in O_2 uptake (results not shown), though State 4 mitochondrial NAD(P) became more reduced. Moreover, the response of mitochondrial NAD(P) to addition of ADP was that of reduction (Figs. 9*c* and 9*d*) rather than oxidation. The marked reduction of mitochondrial NAD(P) consequent on addition of ADP in the prior presence of oligomycin and ATP (Figs. 9*c* and 9*d*) is consistent with activation of 2-oxoglutarate dehydrogenase (McCormack & Denton, 1979) and inhibition of pyruvate dehydrogenase kinase (Hucho *et al.*, 1972) by ADP. ATP was included in the incubation medium in the experiments involving oligomycin to avoid depletion of the pool of intramitochondrial nucleotide. A synergistic effect of Ca^{2+} and ADP on 2-oxoglutarate dehydrogenase has been previously noted (Lawlis & Roche, 1981*a*,*b*).

It seems likely that the different responses to carboxy-

atractyloside and oligomycin have their basis in the different modes of control of the adenine nucleotide translocase and the ATP synthase. Addition of Ca^{2+}, at non-saturating concentrations of pyruvate and 2-oxoglutarate, gives rise to higher values of mitochondrial NAD(P)H (Figs. 3 and 5), as a consequence of dehydrogenase activation. The higher NAD(P)H content would be expected to be associated with greater values of protonmotive force (Mitchell, 1979) and therefore greater rates of ATP synthesis (see Duszynski *et al.*, 1984).

In the presence of the pseudo-irreversible inhibitor oligomycin, the increased matrix ratio of ATP/ADP, which would be expected owing to substrate-level phosphorylation and the presence of extramitochondrial ATP, may inhibit flux through the remaining active ATP synthase molecules, as ATP and ADP are known to compete for the same sites on this enzyme (Krull & Schuster, 1981; Clark *et al.*, 1984). The finding of a lack of stimulation by Ca^{2+} of flux through oxidative phosphorylation, when this is inhibited to 50% by oligomycin, implies that there is no activation of the ATP synthase by Ca^{2+} which is capable of reversing inhibition by a high ATP/ADP ratio and oligomycin. By contrast, when the ATP synthase is uninhibited but the adenine nucleotide translocase is titrated to 50% activity with carboxyatractyloside, an increased matrix ATP/ADP ratio, occurring in response to dehydrogenase activation and an elevated NAD(P)H content, might well give rise to more flux through the uninhibited translocase molecules, in view of the known competition between ATP and ADP for translocation (see Klingenberg, 1976).

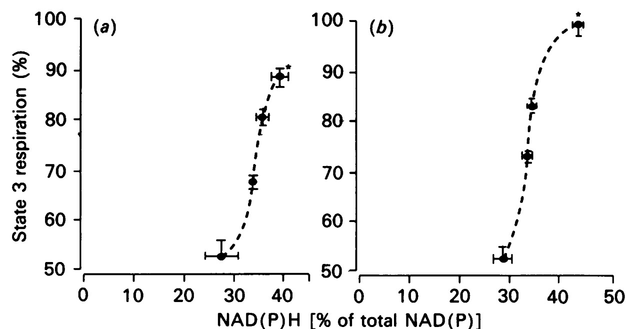

Fig. 8. Relationship between NAD(P)H and 50% State 3 respiration in carboxyatractyloside-treated mitochondria

Mitochondria (1.2–1.6 mg of protein/ml) were incubated with the indicated oxidizable substrates in the presence of carboxyatractyloside [373 ± 54 pmol/mg of protein (n = 5) and 343 ± 36 pmol/mg of protein (n = 5) for 0.5 mM-pyruvate + 1 mM-malate (*a*) and 1 mM-2-oxoglutarate (*b*) respectively]. Then the rate of State 3 respiration and the level of NAD(P)H were varied by adding different Ca^{2+}/EGTA buffers to stabilize 5 nM (initial value), 66, 130 and 225 nM free Ca^{2+}. The rate of State 3 respiration was normalized to the rate obtained in the absence of carboxyatractyloside, i.e. 181 ± 19 and 144 ± 13 ng-atoms of O_2/min per mg (means ± S.E.M., n = 5) for 0.5 mM-pyruvate + 1 mM-malate and 1 mM-2-oxoglutarate respectively. * Values of % NAD(P)H at 5 and 225 nM-Ca^{2+} are significantly different with both pyruvate plus malate ($P < 0.05$) and 2-oxoglutarate ($P < 0.01$) as substrate. Rates of State 3 respiration are also significantly different ($P < 0.001$) at 5 and 225 nM-Ca^{2+}, for both substrates.

Conclusions

At low concentrations of NAD-linked substrates, dehydrogenase activity clearly plays a major role in the limitation of flux through oxidative phosphorylation (Figures 2, 4, 6 and 7). It is noted that physiological concentrations of pyruvate, 2-oxoglutarate and glutamate normally fall within this range, where they would be expected to be a determinant of respiratory activity; free concentrations of long-chain acyl carnitine species *in vivo* are difficult to estimate. These results are not surprising, given the non-limiting availability of ADP and P_i in the present (State 3) experiments. However, the experiments with carboxyatractyloside (Fig. 8) reproduced, by an artificial means, the limiting rate of ADP supply to the mitochondrial ATP synthase that is probably seen *in vivo*. It is significant that

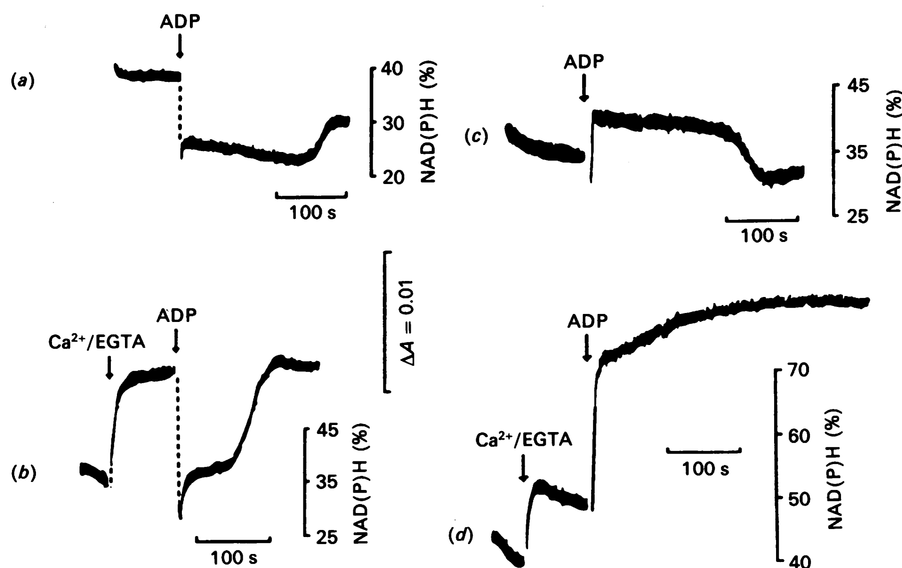

Fig. 9. Redox state of mitochondria during 50% State 3 oxidation of 2-oxoglutarate

Mitochondria (1.4 mg of protein/ml for *a* and *b*; 1 mg of protein/ml for *c* and *d*) were incubated with 1 mM-2-oxoglutarate in the presence of 360 pmol of carboxyatractyloside (*a* and *b*) or 450 pmol of oligomycin (*c* and *d*) per mg of protein to give 50% inhibition of State 3 respiration. Other additions were 225 nM free Ca^{2+} and 1 mM-ADP, after 2 and 5 min of incubation respectively. The standard medium was supplemented with 1 mM-ATP for the experiments with oligomycin (*c* and *d*). The level of NAD(P)H was determined as described in the legend to Fig. 1.

under these conditions dehydrogenase activation by Ca^{2+} can generate increased flux through oxidative phosphorylation (Fig. 8). This is entirely consistent with the point of view expressed, for example by Wilson *et al.* (1977) and van der Meer *et al.* (1978), that both $NADH/NAD^+_{mito}$ and $ATP/ADP \times P_i$ terms are involved in determining flux through oxidative phosphorylation. Koretsky & Balaban (1987) have also emphasized the role of the $NADH/NAD^+$ ratio in experiments with liver mitochondria using different oxidizable substrates to generate different values of $NADH/NAD^+$ and State 3 rates of O_2 uptake. The present study supports and extends their findings by using a wider range of substrate conditions and by altering ADP availability.

We are aware that the magnitude of the changes in the $NADH/NAD^+$ ratio which occur as a consequence of varying Ca^{2+} or substrate availability in State 3 is not large (10–20% in Figs. 2, 4, 6 and 7). The model of oxidative phosphorylation which we are examining, in common with that of Koretsky & Balaban (1987), requires that the extra flux through oxidative phosphorylation at higher substrate or Ca^{2+} concentration be driven by the greater free energy change of the redox reactions of the respiratory chain, or ΔE_h. It would be speculative to argue about the adequacy of the changes in the $NADH/NAD^+$ ratio determined here, as the flux is considered more properly to relate to the disequilibrium between the ΔE_h of the respiratory chain and the protonmotive force, $\Delta\bar{\mu}_{H^+}$ (Mitchell, 1966, 1979; reviewed in Hansford, 1980). Values of $\Delta\bar{\mu}_{H^+}$ are not available for the experiments presented here. However, there is a rather general finding of a very steep dependency of flux through oxidative phosphorylation on $\Delta\bar{\mu}_{H^+}$ in State 3 mitochondria (Azzone *et al.*, 1978; Woelders *et al.*, 1988).

At the low extreme of ADP availability (State 4), we have shown that respiration was significantly increased by increased substrate concentration or by Ca^{2+} (see Figs. 2, 4, 6 and 7). As the main controlling step in State 4 respiration is proton permeation through the mitochondrial inner membrane (Groen *et al.*, 1982; Moreno-Sánchez, 1985*b*; Brand *et al.*, 1988) it seems likely that increased protonmotive forces associated with increased dehydrogenase activity are driving increased flux of protons; this effect can become more pronounced when protonmotive forces are high enough that membrane conductance to protons become non-ohmic (Nicholls, 1974).

If in the present work Ca^{2+} ions were functioning solely to activate pyruvate, NAD-isocitrate and 2-oxoglutarate dehydrogenases (for reviews see Hansford, 1985; Denton & McCormack, 1985), the experimental points obtained with Ca^{2+} would superimpose on those obtained by varying the substrate concentration. With pyruvate (Fig. 4), 2-oxoglutarate (Fig. 6) and glutamate plus malate (Fig. 7), there is a clear displacement of the two curves. Clearly Ca^{2+} is acting to activate dehydrogenases, as seen by the effect of increased reduction of NAD(P) in Figs. 3, 5 and 9. However, the very steep relationship between State 3 respiration and percentage reduction of NAD(P) in the presence of Ca^{2+} (Figs. 4, 6 and 7) suggests that Ca^{2+} is also activating oxidative phosphorylation *per se*, i.e. the oxidation of NADH by the respiratory chain, coupled to phosphorylation of ADP. This is consistent with earlier work (Moreno-Sánchez, 1985*a*,*b*) in which an activation by Ca^{2+} was noted in a system which shows no effects of Ca^{2+} on dehydrogenase levels, i.e. oxidative phosphorylation with succinate as substrate, in the presence of rotenone. It is possible that Ca^{2+} can induce a transition of the ATP synthase from an inactive to an active state either through affecting the degree of association of the low molecular mass inhibitor protein with the enzyme (Tuena de Gomez-Puyou *et al.*, 1980; Gomez-Puyou *et al.*, 1983; Yamada & Huzel, 1983, 1988) or by changing the kinetic properties of the ATP synthase (Matsuno-Yagi & Hatefi, 1986).

Alternatively, Ca^{2+} may be acting at the level of the adenine nucleotide translocase. This indeed would be an attractive explanation of the steep dependence of flux on the $NADH/NAD^+$ ratio obtained when Ca^{2+} is used to activate oxidative phosphorylation, in the presence of a translocase made more limiting by partial inhibition with carboxyatractyloside (Fig. 8). It would also be consistent with the lack of effect of Ca^{2+} on oxidative arsenylation, a process which does not require flux through the adenine nucleotide translocase (Moreno-Sánchez, 1985*b*), and the lack of effect of Ca^{2+} in the present study when oligomycin was used to make the ATP synthase more limiting. Stimulation of the resolved and reconstituted adenine nucleotide translocase by Ca^{2+} has been reported (Kramer *et al.*, 1986) but the K_a was approx. 10^{-4} M, such that the significance to the present studies is not clear.

Finally, Ca^{2+} may be acting at the level of the respiratory chain, and a mechanism whereby Ca^{2+} enhances mitochondrial pyrophosphate content, matrix volume and respiratory chain activity has been suggested by Halestrap (1989). Studies by Halestrap (1987) on heart mitochondria have shown that these Ca^{2+}-induced volume changes, which correlate with stimulation of respiration, are quite slow, requiring some 2 min for completion. By contrast, the activation of dehydrogenases upon addition of Ca^{2+} to heart mitochondria is relatively rapid, being largely complete within 30 s as judged from studies of mitochondrial NADH content (Hansford & Castro, 1981; present paper, Figs. 3*b*, 5*b*, 9*b*). In the present study, Ca^{2+} was added 3 min prior to addition of ADP, allowing volume-dependent effects of the sort described by Halestrap (1987) to develop: challenge with ADP after a shorter exposure to Ca^{2+} might yield information on the interplay of these effects at the level of the dehydrogenases and, potentially, at the level of the respiratory chain.

REFERENCES

Assimacopoulos-Jeannet, F., McCormack, J. G. & Jeanrenaud, R. (1983) FEBS Lett. **159**, 83–88
Azzone, G. F., Pozzan, T., Massari, S. & Bragadin, M. (1978) Biochim. Biophys. Acta **501**, 296–306
Baggetto, L., Gautheron, D. C. & Godinot, C. (1984) Arch. Biochem. Biophys. **232**, 670–678
Balaban, R. S. & Blum, J. J. (1982) Am. J. Physiol. **242**, C172–C177
Balaban, R. S., Kantor, H. L., Katz, L. A. & Briggs, R. W. (1986) Science **232**, 1121–1123
Bohnensack, R., Kuster, U. & Letko, G. (1982) Biochim. Biophys. Acta **680**, 271–280
Brand, M. D., Hafner, R. P. & Brown, G. C. (1988) Biochem. J. **255**, 535–539
Bünger, R. & Permanetter, B. (1984) Am. J. Physiol. **247**, C45–C52
Chance, B. & Williams, G. R. (1955) J. Biol. Chem. **217**, 409–427
Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) J. Biol. Chem. **258**, 8769–8773
Clark, D. D., Daggett, S. G. & Schuster, S. M. (1984) Arch. Biochem. Biophys. **233**, 378–392
Crompton, M. (1982) Curr. Top. Membr. Transp. **25**, 231–276
Davis, E. J. & Davis-Van Thienen, W. I. A. (1984) Arch. Biochem. Biophys. **233**, 573–581
Denton, R. M. & McCormack, J. G. (1985) Am. J. Physiol. **249**, E543–E554
Denton, R. M., Richards, D. A. & Chin, J. G. (1978) Biochem. J. **176**, 899–906
Doussiere, J., Ligeti, E., Brandolin, G. & Vignais, P. V. (1984) Biochim. Biophys. Acta **766**, 492–500
Duszynski, J., Bogucka, K. & Wojtczak, Z. (1984) Biochim. Biophys. Acta **767**, 540–547
From, A. H. L., Petein, M. A., Michurski, S. P., Zimmer, S. D. & Ugurbil, K. (1986) FEBS Lett. **206**, 257–261
Gomez-Puyou, A., Ayala, G., Muller, U. & Tuena de Gomez-Puyou, M. (1983) J. Biol. Chem. **258**, 13673–13679

Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., Van der Meer, R. & Tager, J. M. (1982) J. Biol. Chem. **257**, 2754–2757
Halestrap, A. P. (1987) Biochem. J. **244**, 159–164
Halestrap, A. P. (1989) Biochim. Biophys. Acta **973**, 355–382
Hansford, R. G. (1980) Curr. Top. Bioenerg. **10**, 217–278
Hansford, R. G. (1985) Rev. Physiol. Biochem. Pharmacol. **102**, 1–72
Hansford, R. G. & Castro, F. (1981) Biochem. J. **198**, 525–533
Hansford, R. G. & Johnson, R. N. (1975) J. Biol. Chem. **250**, 8361–8375
Heinrich, R. & Rapoport, T. A. (1974) Eur. J. Biochem. **42**, 89–95
Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W. & Reed, L. J. (1972) Arch. Biochem. Biophys. **151**, 328–340
Kacser, H. & Burns, J. A. (1973) Symp. Soc. Exp. Biol. **27**, 65–104
Kantor, H. L., Briggs, R. W., Metz, K. R. & Balaban, R. S. (1986) Am. J. Physiol. **251**, H171–H175
Katz, L. A., Koretsky, A. P. & Balaban, R. S. (1987) FEBS Lett. **221**, 270–276
Katz, L. A., Koretsky, A. P. & Balaban, R. S. (1988) Am. J. Physiol. **255**, H185–H188
Katz, L. A., Swain, J. A., Portman, M. A. & Balaban, R. S. (1989) Am. J. Physiol. **256**, H265–H274
Khym, J. X. (1975) Clin. Chem. **21**, 1245–1252
Klingenberg, M. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), vol. 3, pp. 383–438, Plenum, New York
Koretsky, A. P. & Balaban, R. S. (1987) Biochim. Biophys. Acta **893**, 398–408
Kramer, R., Mayr, U., Heberger, C. & Tsompanidou, S. (1986) Biochim. Biophys. Acta **855**, 201–210
Krull, K. W. & Schuster, S. M. (1981) Biochemistry **20**, 1592–1598
Kurlansky, S. B., Hilburger, A. C. & Levy, H. R. (1988) Arch. Biochem. Biophys. **264**, 93–102
La Noue, K., Niklas, W. J. & Williamson, J. R. (1970) J. Biol. Chem. **245**, 102–111
Lawlis, V. B. & Roche, T. E. (1981*a*) Biochemistry **20**, 2512–2518
Lawlis, W. B. & Roche, T. E. (1981*b*) Biochemistry **20**, 2519–2524

Received 16 October 1989/7 February 1990; accepted 19 February 1990

Matsuno-Yagi, A. & Hatefi, Y. (1986) J. Biol. Chem. **261**, 14031–14038
McCormack, J. G. & Denton, R. M. (1979) Biochem. J. **180**, 533–544
McCormack, J. G., Browne, H. M. & Dawes, N. J. (1989) Biochim. Biophys. Acta **973**, 420–427
Mitchell, P. (1966) Biol. Rev. **41**, 445–502
Mitchell, P. (1979) Eur. J. Biochem. **95**, 1–20
Moreno-Sánchez, R. (1985*a*) J. Biol. Chem. **260**, 4028–4034
Moreno-Sánchez, R. (1985*b*) J. Biol. Chem. **260**, 12554–12560
Moreno-Sánchez, R. & Hansford, R. G. (1988) Biochem. J. **256**, 403–412
Moreno-Sánchez, R., Hogue, B. A. & Hansford, R. G. (1989) FASEB J. **3**, A703
Nicholls, D. G. (1974) Eur. J. Biochem. **50**, 305–315
Staddon, J. M. & Hansford, R. G. (1986) Biochem. J. **238**, 737–743
Staddon, J. M. & Hansford, R. G. (1987) Biochem. J. **241**, 729–735
Sugden, M. C., Ball, A. J. & Williamson, D. H. (1980) Biochem. Soc. Trans. **8**, 591–592
Tuena de Gomez-Puyou, M., Gauilanes, M., Gomez-Puyou, A. & Ernster, L. (1980) Biochim. Biophys. Acta **592**, 396–405
van der Meer, R., Akerboom, T. P. M., Groen, A. K. & Tager, J. M. (1978) Eur. J. Biochem. **84**, 421–428
Vinogradov, A., Scarpa, A. & Chance, B. (1972) Arch. Biochem. Biophys. **152**, 646–654
Wan, B., LaNoue, K. F., Cheung, J. Y. & Scaduto, R. C. (1989) J. Biol. Chem. **264**, 13430–13439
Weisner, R. J., Kreutzer, U., Rosen, P. & Grieshaber, M. K. (1988) Biochim. Biophys. Acta **936**, 114–123
Williamson, J. R. (1979) Annu. Rev. Physiol. **41**, 485–506
Williamson, J. R. & Corkey, B. E. (1969) Methods Enzymol. **13**, 434–513
Wilson, D. F., Owen, C. S. & Holian, A. (1977) Arch. Biochem. Biophys. **182**, 749–762
Woelders, H., van der Velden, T. & van Dam, K. (1988) Biochim. Biophys. Acta. **934**, 123–134
Yamada, E. W. & Huzel, N. J. (1983) Biosci. Rep. **3**, 947–954
Yamada, E. W. & Huzel, N. J. (1988) J. Biol. Chem. **263**, 11498–11503