# Control of the effective P/O ratio of oxidative phosphorylation in liver mitochondria and hepatocytes

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The control exerted by substrate oxidation reactions, by ATP turnover and by the proton leak over the oxygen consumption rate, the phosphorylation rate, the proton leak rate and the protonmotive force ($\Delta p$) in isolated rat liver mitochondria under a range of conditions between non-phosphorylating (State 4) and maximum phosphorylation (State 3) was investigated by using the top-down approach of metabolic control analysis. The experiments were carried out with saturating concentrations of the substrates succinate, glutamate with malate, or pyruvate with malate. The distribution of control was very similar with each of the three substrates. The effective P/O ratio (i.e. not corrected for leak reactions) was also measured; it varied from zero in State 4 to 80–90% of the maximum theoretical P/O ratio in State 3. Under most conditions control over the effective P/O ratio was shared between proton leak (which had negative control) and the phosphorylating subsystem (which had roughly equal positive control); near State 4, substrate oxidation reactions also acquired some control over this ratio. In resting hepatocytes the effective P/O ratio was only 50% of its maximum theoretical value, corresponding to an effective P/O ratio of only 1.3 for complete oxidation of glucose. The effective P/O ratio for intracellular mitochondrial oxygen consumption was 64% of the maximum value. The control coefficient of the mitochondrial proton leak over the effective P/O ratio in cells was −0.34; the control coefficient of phosphorylation reactions over this ratio was 0.31 and the control coefficient of substrate oxidation reactions over the ratio was 0.03, showing how the coupling efficiency in cells can respond sensitively to agents that change the proton leak or the ATP demand, but not to those that change substrate oxidation.

## INTRODUCTION

Metabolic control analysis (Heinrich and Rapoport, 1974; Kacser and Burns, 1979) is well suited to the study of the control of oxidative phosphorylation, since there are many specific and potent inhibitors available that can be used to titrate particular steps and so allow direct calculation of flux control coefficients (Groen et al., 1982; Tager et al., 1983; Brand and Murphy, 1987). It was first used in this context by Groen et al. (1982), who showed how the control over respiration rate of rat liver mitochondria exerted by particular reactions varied with the rate of oxidative phosphorylation between State 4 and State 3. Subsequent work by several different groups has confirmed and extended the original analysis (Brand and Murphy, 1987; Fell, 1992).

However, the approach of using specific inhibitors fails when it is applied to one of the important reactions of oxidative phosphorylation, i.e. the leak of protons across the mitochondrial inner membrane, since no specific inhibitor is available. In early work, uncouplers were used to increase the leak and so allow measurement of its flux control coefficient (Groen et al., 1982), but this procedure has been shown to be theoretically invalid and should not be used (Brand et al., 1988). An equally simple but more successful method is to measure the elasticity (i.e. the kinetic response) of the leak and of the rest of the system to the protonmotive force ($\Delta p$) and to calculate the flux control coefficient from the elasticities by using connectivity and summation relationships (Brand et al., 1988). This 'top-down' approach to metabolic control analysis (Brand et al., 1988; Brown et al., 1990a) is used in the present paper; it simplifies the metabolic system under consideration to the blocks of reactions that produce a defined intermediate and those that consume it. In the case of respiration in isolated mitochondria a suitable intermediate is $\Delta p$, or the mitochondrial membrane potential, $\Delta\psi$. The intermediate is produced by the reactions of 'substrate oxidation', S (which includes the substrate carriers, dehydrogenases and other enzymes, and the electron-transport chain). The intermediate is consumed by two blocks of reactions: the 'phosphorylating subsystem', P (which includes the ATP synthase, the phosphate carrier, the adenine nucleotide carrier and any reactions that may be present that convert ATP into ADP), and the 'proton leak', L (which consists of all reactions that consume $\Delta p$ without ATP synthesis, but mainly represents the leak of protons across the mitochondrial inner membrane). An ADP-regenerating system consisting of hexokinase, glucose and phosphate can be used to generate a range of conditions between no phosphorylation (State 4) and maximum rate of phosphorylation (State 3).

Hafner et al. (1990) used this top-down approach to analyse the control of respiration rate, phosphorylation rate, proton leak rate and $\Delta p$ in isolated rat liver mitochondria using succinate as the substrate. The results were compatible with and comp-

Abbreviations used: S, substrate oxidation subsystem; P, phosphorylating subsystem; L, proton leak; $J_S$, rate of mitochondrial or cellular oxygen consumption; $J_P$, rate of oxygen consumption required to pump protons out of the matrix at a rate equal to their rate of return through the phosphorylating subsystem; $J_L$, rate of oxygen consumption required to pump protons out of the matrix at a rate equal to their rate of return through the proton leak; $\Delta p$, protonmotive force; $\Delta\psi$, transmembrane difference in electrical potential; $\Delta pH$, transmembrane difference in pH; State 3, condition with maximum phosphorylation, with ADP, excess hexokinase and excess substrate present; State 4, non-phosphorylation condition with ADP, excess substrate and oligomycin present; $P/O_{max}$, maximum (or mechanistic) P/O ratio, maximum possible mol of ATP produced per mol of O consumed for a particular substrate; effective P/O ratio, actual mol of ATP produced per mol of O consumed, with no correction for incomplete coupling; $C_E^A$, overall control coefficient of step E over flux, concentration or ratio A.

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lementary to those obtained by Groen et al. (1982), who analysed the control of respiration rate by examining the effects of inhibiting each enzyme in the pathway individually ('bottom-up' control analysis), except that top-down control analysis allowed the control by the proton leak to be correctly measured. Top-down control analysis has also been applied to oxidative phosphorylation in hepatocytes (Brown et al., 1990b), where the distribution of control was found to be similar to that in isolated liver mitochondria respiring at intermediate rates and to oxidative phosphorylation in potato tuber mitochondria (Kesseler et al., 1992).

The substrates used *in vivo* by liver mitochondria are fatty acids (especially under conditions of starvation) and NAD-linked substrates such as pyruvate from the breakdown of glucose or fructose. In the present paper we analyse the control of oxidative phosphorylation in isolated mitochondria with substrates other than succinate: glutamate with malate and pyruvate with malate.

Although $P/O_{max.}$, the maximum value of the P/O ratio, with different substrates in mitochondria has been studied for many years, it is only recently that some sort of agreement has been reached about its magnitude (Hinkle et al., 1991); even now not all investigators would agree with the values assumed in the present paper. Rather less attention has been paid to the effective value of the P/O ratio under physiological conditions, in other words the ratio obtained without making allowance for the leaks and inefficiencies in the coupling mechanism, even though it is this ratio that is physiologically more important. There are still papers published that assume that the classical uncorrected maximum value of 3.0 for the P/O ratio of NAD-linked substrates is the relevant value in intact cells and tissues. However, not only is this value now known to be too high for $P/O_{max.}$ (Hinkle et al., 1991), but it has been recognized for many years that the effective P/O ratio in cells will be less than $P/O_{max.}$ because of oxygen consumption not coupled to ATP synthesis (Ernster and Nordenbrand, 1974). We have previously shown that the leak of protons across the mitochondrial inner membrane is not an artefact of isolation, but occurs in intact hepatocytes, where 25–30% of the resting respiration rate is used to drive a futile cycle of proton pumping and proton leak across the mitochondrial inner membrane (Nobes et al., 1990a; Brown et al., 1990b; Harper and Brand, 1993). A further 15–20% is used for non-mitochondrial reactions, so as little as 50% of the oxygen consumption in these resting cells may be associated with ATP synthesis. In the present paper we measure the effective P/O ratio at different respiration rates between State 4 and State 3 in isolated mitochondria respiring on different substrates and assess its value in resting hepatocytes.

Although the theory for calculating the control coefficients over the ratio of two fluxes, such as over the effective P/O ratio, has been worked out (Westerhoff and van Dam, 1987), no complete study of the control of this ratio has been published either for mitochondria respiring at intermediate rates between State 3 and State 4 or for whole cells. In the present paper we conduct such an analysis and show how the value of the effective P/O ratio can be rather sensitive to agents that alter the proton leak or ATP demand, but insensitive to agents that affect substrate oxidation, both in isolated mitochondria and in hepatocytes.

## MATERIALS AND METHODS

### Isolation of mitochondria

Mitochondria were prepared by standard procedures (Chappell and Hansford, 1972) from female rats (approx. 250 g) in a medium containing 250 mM sucrose, 5 mM Tris and 1 mM EGTA, adjusted to pH 7.4 with HCl. Mitochondrial protein was assayed by a biuret method (Gornall et al., 1949) with BSA as standard.

### Measurement of oxygen consumption and Δp

Mitochondrial respiration rate was measured with an oxygen electrode. Air-saturated medium at 37 °C was assumed to contain 440 nmol of O/ml. Mitochondrial membrane potential ($\Delta\psi$) was measured with an electrode sensitive to methyltriphenylphosphonium cation ($TPMP^+$) as described by Brown and Brand (1985). This was coupled to a reference electrode, and both electrodes were inserted through an air-tight port into the oxygen-electrode vessel to monitor oxygen consumption and $\Delta\psi$ simultaneously. The $TPMP^+$ electrode was calibrated for each incubation with 1 μM additions of $TPMP^+$ up to 5 μM. A mitochondrial volume of 0.6 μl/mg of protein (Brown and Brand, 1988) and a binding correction factor for $TPMP^+$ of 0.4 (Brown and Brand, 1985) were assumed. $\Delta\psi$ was calculated as $61.5 \times \log(0.4 \times \text{accumulation ratio of } TPMP^+)$, where accumulation ratio is $[TPMP]_{matrix}/[TPMP]_{medium}$.

Incubations were carried out at 37 °C in 3.3 ml of medium containing 120 mM KCl, 5 mM $KH_2PO_4$, 100 μM ADP, 5 mM $MgCl_2$, 20 mM glucose, 1 mM EGTA and 3 mM Hepes, pH 7.2. For mitochondria (1 mg of protein/ml) oxidizing succinate (10 mM) the medium also contained 5 μM rotenone. For the other substrates, mitochondria (2 mg of protein/ml) were incubated with 4 mM glutamate and 1 mM malate or 5 mM pyruvate and 1 mM malate. All these substrates were in excess; doubling their concentration did not increase the rate of respiration. Titrations with hexokinase were from 0 to 1.25 units/ml. State 4 was achieved by adding 5 μg of oligomycin/mg of protein. Titrations with malonate were up to 6 mM. Titrations with KCN were up to 240 μM. When used, nigericin was present at 80 ng/ml.

ΔpH was determined from the distribution of [1-$^3$H]acetate and [U-$^{14}$C]sucrose, and mitochondrial volume was determined from the distribution of $^3H_2O$ and [$^{14}$C]sucrose as described by Brown and Brand (1985, 1988). Mitochondrial matrix volume was measured with 0.5 μCi of [$^{14}$C]sucrose/ml for extramitochondrial volume and 1 μCi of $^3H_2O$/ml for total pellet volume. Matrix volume was calculated as $^3H_2O$ space − [$^{14}$C]sucrose space. ΔpH was calculated from the accumulation ratio of [$^3$H]acetate. Accumulation ratio of acetate was calculated as ([$^3$H]acetate space − [$^{14}$C]sucrose space)/matrix volume. ΔpH was calculated as $61.5 \times \log$ (acetate accumulation ratio).

Experiments using radioisotopes were done in parallel with assays of the rate of oxygen consumption and $\Delta\psi$; the same media and conditions were used, but 100 μM sodium acetate and radiochemicals were added. The mitochondria were collected by centrifugation for 1 min in a bench microcentrifuge, and the radioactivity of the supernatants and pellets was determined with an LKB Rackbeta liquid-scintillation counter with appropriate corrections for quench and cross-over.

### Control coefficients over effective P/O ratios

The effective P/O ratio is the amount of ATP produced per total O consumed, and so decreases as the leak reactions increase; in the presence of oligomycin there is no phosphorylation and the effective P/O ratio is zero. This distinguishes it from the mechanistic or maximum P/O ratio, which is the theoretical maximum ratio when leak reactions are zero (or are subtracted).

Maximum P/O ratios were taken to be 1.5 for oxidation of

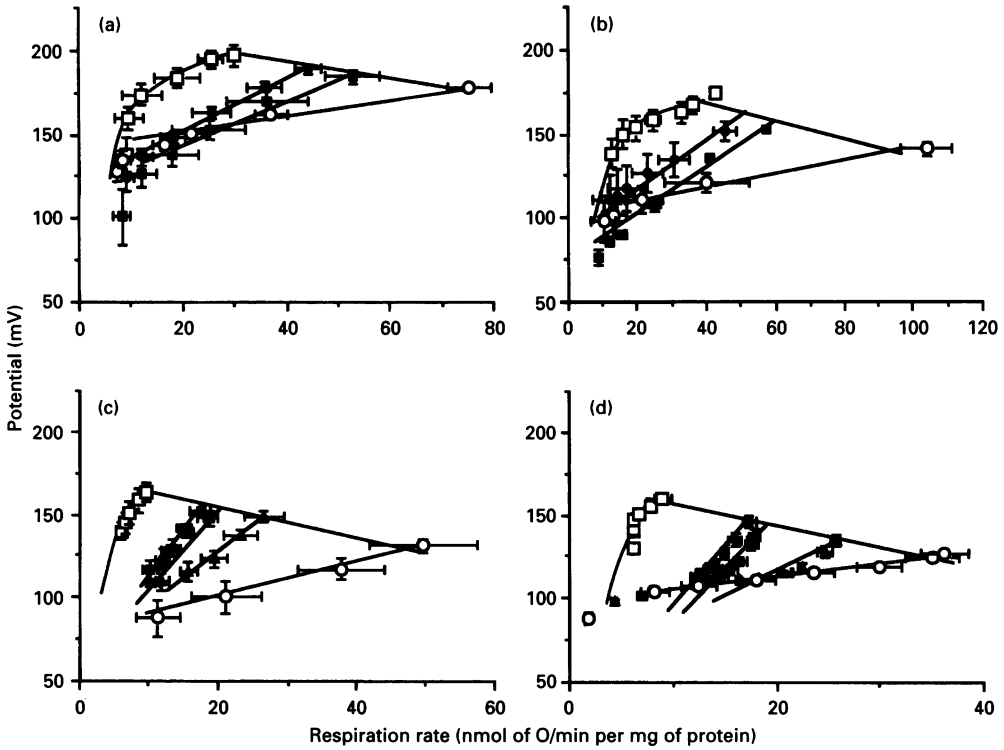


**Figure 1 Measurement of $J_S$, $J_P$ and $J_L$ and the elasticities to $\Delta\psi$ of $J_S$, $J_P$ and $J_L$ in isolated rat liver mitochondria**

Membrane potential and respiration rate were measured with mitochondria oxidizing (**a**) succinate in the presence of nigericin, (**b**) succinate in the absence of nigericin, (**c**) glutamate with malate, or (**d**) pyruvate with malate. Mitochondria were incubated and titrated with hexokinase and malonate or KCN as described in the Materials and methods section. The titrations with respiratory-chain inhibitor in the presence of oligomycin (State 4) (□) give both $J_L$ and the elasticity of the proton leak to $\Delta\psi$ at any given $\Delta\psi$. The elasticity is the normalized slope of the tangent to the curve at that $\Delta\psi$. The titrations with respiratory inhibitor in State 3 (○) and at intermediate respiration rates achieved by adding different concentrations of hexokinase (●, ▲, ■), after subtraction (not shown) of the State 4 titrations to correct for proton leak, give both $J_P$ and the elasticity of the phosphorylating system to $\Delta\psi$ at any given $\Delta\psi$. The lines through the highest points for each of the titration curves represent titrations of State 4 with hexokinase and give both $J_S$ and the elasticity of substrate oxidation to $\Delta\psi$ at any given $\Delta\psi$. Points in (**a**) and (**c**) represent the mean ± S.E.M. for duplicate determinations on three separate preparations. Points in (**b**) and (**d**) represent the mean and range for duplicate determinations on two separate preparations. Lines were drawn by eye and were used to calculate elasticities and control coefficients.

succinate to malate, 2.45 for complete oxidation of pyruvate with malate present and 2.33 for oxidation of malate with glutamate present. These values were calculated by using the now widely accepted $H^+/O$ stoichiometries of 10 for oxidation of NAD-linked substrates and 6 for oxidation of succinate and an $H^+/ATP$ stoichiometry of 4 for synthesis and export of ATP (Hinkle and Yu, 1979; Stoner, 1987; Hinkle et al., 1991). For succinate oxidation 6 protons are pumped per oxygen and four are used per ATP synthesized, so $P/O_{max.}$ is $6/4 = 1.5$. For complete oxidation of pyruvate 46 protons are pumped; one ATP equivalent is synthesized and then exported by using one proton, and five oxygen atoms are consumed, so $P/O_{max.}$ is $[(46-1)/4+1]/5 = 2.45$. For oxidation of glutamate plus malate, 26 protons are pumped; one proton is used for glutamate/aspartate exchange; one ATP equivalent is synthesized and then exported by using one proton, and three oxygens are consumed during oxidation of 2-oxoglutarate to oxaloacetate, so $P/O_{max.}$ is $[(26-2)/4+1]/3 = 2.33$. Since phosphorylation flux in the present paper is measured as the oxygen consumption used to drive the phosphorylation system, the flow ratio of $J_P$ divided by $J_S$ gives the fraction of oxygen consumption that is used to drive phosphorylation (regardless of the true value of the maximum P/O ratio); the remaining fraction of the oxygen consumption ($J_L/J_S$) is used to drive the proton leak. The effective P/O ratio at any rate between State 3 and State 4 is then this flow ratio multiplied by the maximum P/O ratio, i.e.

$$\text{effective P/O ratio} = P/O_{max.} \cdot J_P/J_S$$

It is straightforward to show that the control coefficient of a reaction or a block of reactions over the ratio of two fluxes in a branched system is given by the difference between the control coefficients over the two fluxes. Thus the flux control coefficient of substrate oxidation over the effective P/O ratio is given by

$$C_S^{P/O} = C_S^{J_P} - C_S^{J_S}$$

and similarly the control by the phosphorylation system is given by

$$C_P^{P/O} = C_P^{J_P} - C_P^{J_S}$$

and the control by the proton leak is given by

$$C_L^{P/O} = C_L^{J_P} - C_L^{J_S}$$

[see also equation 4.195 in Westerhoff and van Dam (1987), which describes the control over a ratio of fluxes]. A summation theorem applies: since the three control coefficients over $J_P$ sum to 1 and the three control coefficients over $J_S$ also sum to 1, the control coefficients over the effective P/O ratio sum to the difference, which is 0. Since these control coefficients describe

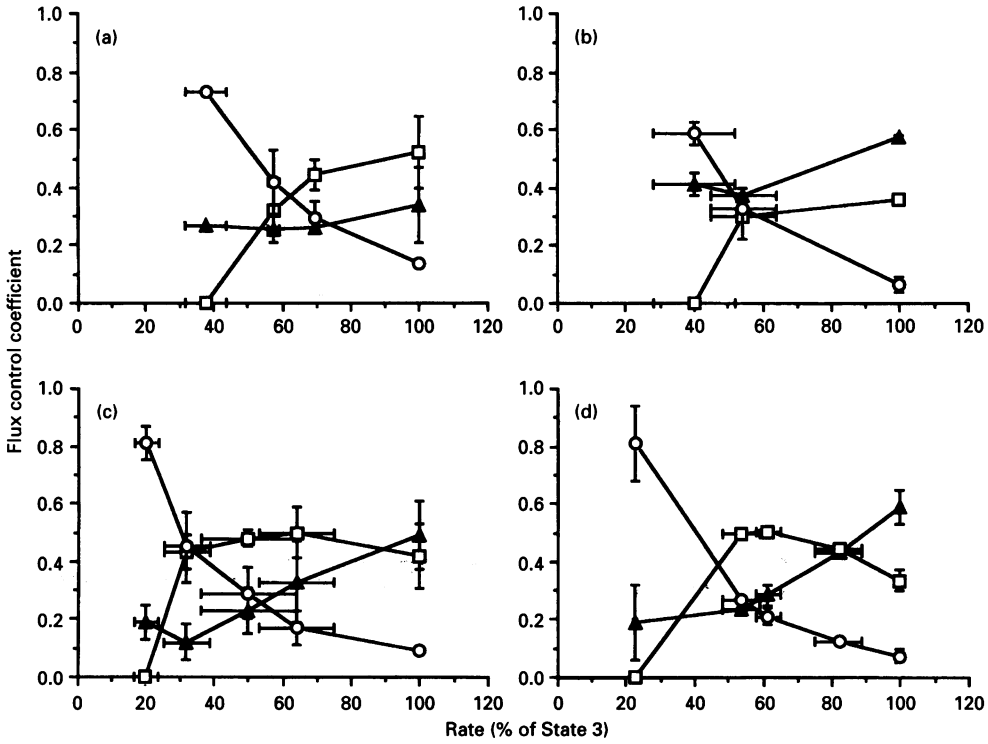


**Figure 2 Overall flux control coefficients of substrate oxidation, proton leak and the phosphorylation system over mitochondrial respiration rate, $J_S$**

Respiration rate is shown as a percentage of the State 3 rate with each substrate. Results are for mitochondria oxidizing (**a**) succinate in the presence of nigericin, (**b**) succinate in the absence of nigericin, (**c**) glutamate with malate, or (**d**) pyruvate with malate. Control coefficients were calculated from the fluxes and elasticities obtained from Figure 1. Key: □, phosphorylation system; ○, proton leak; ▲, substrate oxidation.

control over the fraction of the oxygen flux that is used to drive ATP synthesis (i.e. over the flux ratio $J_P/J_C$), their values are independent of assumed or calculated values of $P/O_{max.}$.

## RESULTS

A series of titrations of $\Delta\psi$ and respiration rate was carried out with isolated mitochondria to determine the fluxes through substrate oxidation ($J_S$), the phosphorylating system ($J_P$) and the proton leak ($J_L$) at different values of $\Delta\psi$ and different respiration rates between State 4 and State 3 (see Brand, 1990). $J_S$ is the total rate of oxygen consumption at any given $\Delta\psi$. $J_L$ at any given $\Delta\psi$ is the rate of oxygen consumption in the presence of excess oligomycin (to prevent phosphorylation) and can be determined over a range of values of $\Delta\psi$ by titrating respiration with a respiratory-chain inhibitor (Brand et al., 1988), such as malonate when the substrate is succinate or KCN for NAD-linked substrates. $J_P$ at any given $\Delta\psi$ is calculated as $J_S - J_L$ at the same $\Delta\psi$. The elasticities of S, P and L to $\Delta\psi$ at any particular value of $\Delta\psi$ are the normalized gradients of plots of the appropriate $J$ against $\Delta\psi$ (see Hafner et al., 1990).

Hafner et al. (1990) also give the equations with which the flux control coefficients and concentration control coefficients can be calculated from the elasticities. Note that $J_P$ is zero in State 4 with oligomycin present and that all terms that contain elasticities of the phosphorylation system to $\Delta p$ in the equations are multiplied by $J_P$; all such terms therefore also become zero. Thus $C^{J_P}_P$ in State 4 is 1.0 and $C^{J_P}_C$ and $C^{J_P}_L$ in State 4 are 0.0. Control coefficients over the effective P/O ratio were calculated as the control coefficient over phosphorylation rate minus the control coefficient over substrate oxidation rate as described in the Materials and methods section.

Titrations were carried out under four different conditions: succinate with nigericin present to collapse $\Delta pH$ and allow the whole of $\Delta p$ to be expressed as $\Delta\psi$; succinate without nigericin; glutamate with malate; and pyruvate with malate. In the case of glutamate with malate or pyruvate with malate, there was no rotenone present and there may have been some oxidation of endogenous substrates. This was not eliminated or quantified, but the respiration before addition of the substrate was much less than when the exogenous substrates were added, which suggests that in these experiments most of the respiration used the added substrates. Nigericin was not used with the NAD-linked substrates, as it inhibited respiration, perhaps because of secondary effects of a relatively acid matrix pH on NADH dehydrogenase. Control experiments suggested that there was less inhibition of State 3 respiration rate by nigericin at alkaline pH than there was at neutral pH (results not shown). Direct and indirect assays showed that $\Delta pH$ was between 20 and 45 mV in all the experiments without nigericin. Analysis of the results showed that the control coefficients with succinate were not particularly different whether nigericin was included or not (see below), nor did addition of 30 mV to the $\Delta\psi$ values significantly change the control coefficients with the NAD-linked substrates. It is valid to ignore any effects of $\Delta pH$ as long as there is a reasonably constant relationship between $\Delta\psi$ and $\Delta pH$ (see Brown et al., 1990a).

The titration curves are shown in Figure 1. The general pattern of the responses was the same, but the absolute values of the

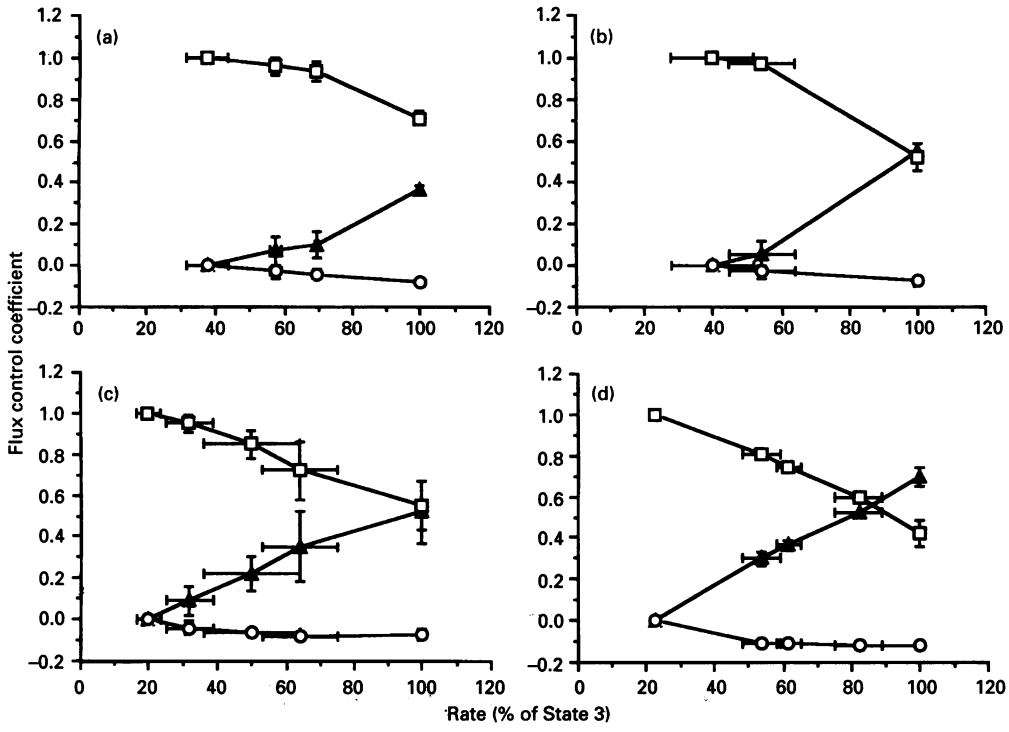


**Figure 3 Overall flux control coefficients of substrate oxidation, proton leak and the phosphorylation system over the phosphorylating system flux, $J_P$**

Respiration rate is shown as a percentage of the State 3 rate with each substrate. Results are for mitochondria oxidizing (**a**) succinate in the presence of nigericin, (**b**) succinate in the absence of nigericin, (**c**) glutamate with malate, or (**d**) pyruvate with malate. Control coefficients were calculated from the fluxes and elasticities obtained from Figure 1. Key: □, phosphorylation system; ○, proton leak; ▲, substrate oxidation.

respiration rates were different with different substrates. The highest membrane potentials occurred during respiration in State 4. This state was titrated in two ways, either (*a*) with an inhibitor of the electron-transport chain, giving lower rates and lower potentials and a curve that represents the rate of proton leak as a function of its 'substrate', $\Delta\psi$, or (*b*) with hexokinase, giving higher rates and lower potentials and a line that represents the rate of the substrate oxidation subsystem as a function of its 'product', $\Delta\psi$. States with excess hexokinase (State 3) or with intermediate concentrations of hexokinase were titrated with an inhibitor of the electron-transport chain, giving lower rates and lower potentials and a set of lines that represent the combined rates of the proton leak and the phosphorylation system as a function of their 'substrate', $\Delta\psi$, under each condition. Subtraction of the titration curve representing the response of the proton leak from each of these titration lines representing the combined response of the proton leak and the phosphorylation system yielded a set of lines that describe the response of the phosphorylation system to $\Delta\psi$ (not shown). Elasticities of $J_S$, $J_P$ and $J_L$ to $\Delta\psi$ were measured as the normalized gradients of the different lines at the appropriate value of $\Delta\psi$. These elasticities and the values of $J_S$, $J_P$ and $J_L$ were used to calculate the control coefficients.

Figure 2 shows the flux control coefficients of substrate oxidation, proton leak and the phosphorylating system over the rate of substrate oxidation, $J_S$, for the four different substrate combinations. The distribution of control was similar in all cases. Under non-phosphorylating conditions (State 4) most (0.6–0.8) of the control resided in the proton leak, with the remainder in substrate oxidation. As respiration was increased by addition of hexokinase towards maximum respiration (State 3), the control by the proton leak fell and the control shifted to substrate oxidation and the phosphorylating system. In State 3 the leak had little control (less than 0.15); the majority of the control was shared about equally between substrate oxidation and the phosphorylation system.

The flux control coefficients of substrate oxidation, proton leak and the phosphorylating system over phosphorylation rate, $J_P$, for the four different substrate combinations are shown in Figure 3. The proton leak had almost negligible, negative control at all rates. At lower rates most of the control lay in the phosphorylating system itself. The control exerted by substrate oxidation increased towards State 3, where it had about half of the control over the rate of phosphorylation.

Figure 4 shows the flux control coefficients of substrate oxidation, proton leak and the phosphorylating system over proton leak rate, $J_L$, for the four different substrate combinations. For all the substrates the leak itself had a strong positive flux control coefficient at all respiration rates. With succinate as substrate (Figures 4a and 4b), substrate oxidation had strong positive control and the phosphorylation system had strong negative control, particularly towards State 3. With NAD-linked substrates (Figures 4c and 4d), the control by these two branches was rather less.

Figure 5 shows the concentration control coefficients of substrate oxidation, proton leak and the phosphorylating system over $\Delta\psi$ for the four different substrate combinations. The pattern of control was similar for each substrate. The values were all small in State 4, indicating that $\Delta\psi$ is fairly stable. Towards State 3, $\Delta\psi$ was mostly controlled by substrate oxidation and the

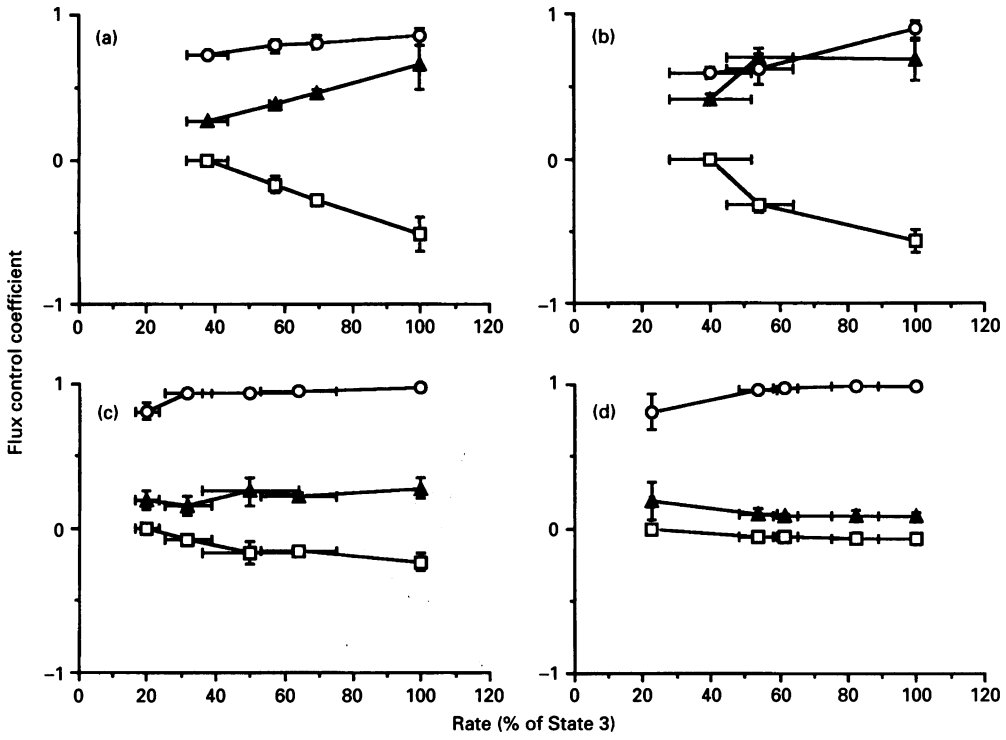


**Figure 4 Overall flux control coefficients of substrate oxidation, proton leak and the phosphorylation system over the proton leak flux, $J_L$**

Respiration rate is shown as a percentage of the State 3 rate with each substrate. Results are for mitochondria oxidizing (**a**) succinate in the presence of nigericin, (**b**) succinate in the absence of nigericin, (**c**) glutamate with malate, or (**d**) pyruvate with malate. Control coefficients were calculated from the fluxes and elasticities obtained from Figure 1. Key: □, phosphorylation system; ○, proton leak; ▲, substrate oxidation.

phosphorylation system, but the coefficients were still fairly small; $\Delta\psi$ is therefore still quite stable even at high rates of respiration.

The fraction of the available proton current that is used for synthesis and export of ATP decreases as the proton leak reactions increase. In State 4 there is no net ATP synthesis; all the proton current is via the leak pathway, and the effective P/O ratio is zero. In State 3 both the absolute flux and the relative flux through the proton leak are less, and the effective P/O ratio is greater, although leaks still occur and the effective P/O ratio is still less than the maximum possible value. Figure 6 shows effective P/O ratios for the different substrates between State 4 and State 3. The scale on the left side gives the flow ratio $J_P/J_S$, which is the fraction of the maximum P/O ratio in that condition. The values are independent of assumptions about the true maximum P/O ratios. The scale on the right converts these fractional values into absolute effective P/O ratios, assuming $P/O_{max.}$ values of 1.5 for succinate, 2.45 for pyruvate with malate and 2.33 for glutamate with malate. With each substrate the effective P/O ratio rose steeply as respiration rate increased above the State 4 rate and the rate of proton leak decreased. However, even near State 3 the effective P/O ratio still changed with respiration rate, showing how the effective coupling ratio of oxidative phosphorylation is a function of ATP demand with all substrates at all respiration rates. This observation confirms that by Ernster and Nordenbrand (1974) and should be contrasted with the lack of effects on the maximum P/O ratio, which remains constant over the whole range of rates and membrane potentials (Hafner and Brand, 1991).

Figure 7 shows the control coefficients of substrate oxidation, proton leak and the phosphorylating system over the effective P/O ratio for the four different substrate combinations. The pattern was the same in each case. Near State 4 the phosphorylation system had a large positive control coefficient, and the proton leak had a large negative coefficient. Substrate oxidation had a small negative coefficient. The control coefficients approached zero as respiration rate increased towards State 3, showing how the effective P/O ratio becomes more stable and is less affected by small changes in the activities of the different parts of the system when ATP turnover is rapid.

Table 1 extends the analysis to rat hepatocytes, using values for $J_P$, $J_S$, $C^{J_P}$ and $C^{J_S}$ from Brown et al. (1990b) and Harper and Brand (1993). In intact cells there is a significant rate of non-mitochondrial oxygen consumption, so the values of the flow ratio $J_P/J_S$ (i.e. the effective P/O ratio as a fraction of $P/O_{max.}$) differ depending on whether this is subtracted or not; the results of both calculations are presented. Values for the flow ratio and for the control coefficients over the P/O ratio were similar in hepatocytes from starved or fed rats, either hypothyroid or euthyroid, so the mean values for fed euthyroid animals will be emphasized here. Because of the presence of proton leak pathways in the cells, the effective P/O ratio based on mitochondrial oxygen consumption was only 64% of $P/O_{max.}$. Theoretically, cells oxidizing glucose produce a maximum of 31 molecules of ATP per molecule of glucose (Hinkle et al., 1991), corresponding to a $P/O_{max.}$ of 2.58. Thus if the hepatocytes in these experiments were completely oxidizing the added glucose as their sole fuel, their effective mitochondrial P/O ratio would be 1.65. The effective P/O ratio based on total oxygen consumption was even smaller, 50% of $P/O_{max.}$, corresponding to an effective P/O ratio of only 1.29.

Table 1 also shows the control coefficients over the effective

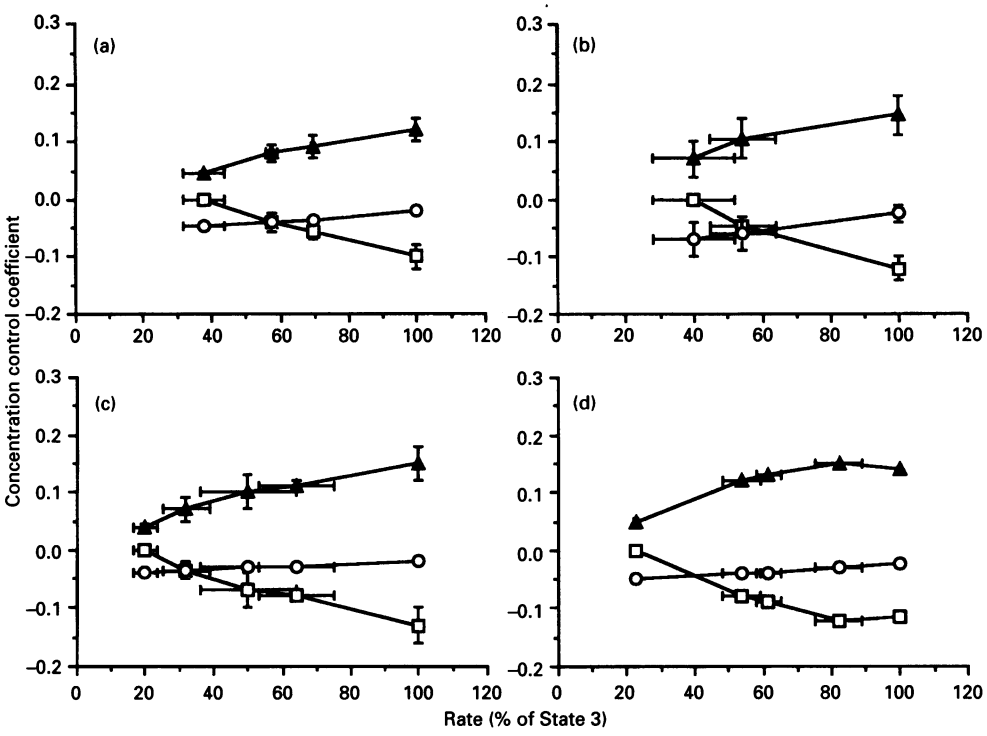


**Figure 5 Overall concentration control coefficients of substrate oxidation, proton leak and the phosphorylation system over $\Delta\psi$**

Respiration rate is shown as a percentage of the State 3 rate with each substrate. Results are for mitochondria oxidizing (**a**) succinate in the presence of nigericin, (**b**) succinate in the absence of nigericin, (**c**) glutamate with malate, or (**d**) pyruvate with malate. Control coefficients were calculated from the fluxes and elasticities obtained from Figure 1. Key: □, phosphorylation system; ○, proton leak; ▲, substrate oxidation.

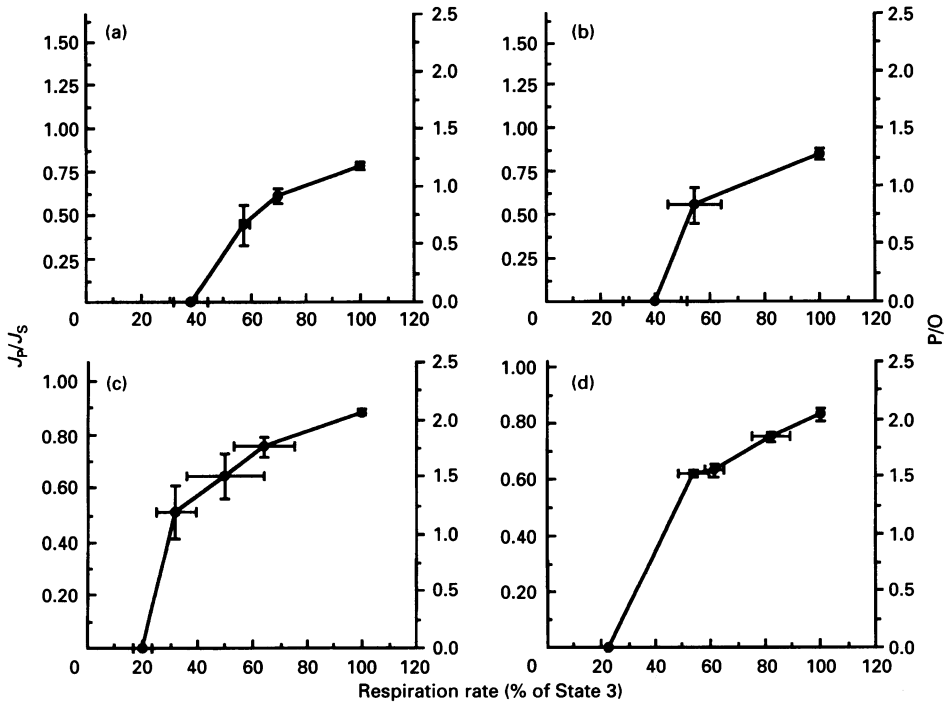


**Figure 6 The effective P/O ratio with different substrates in mitochondria**

Respiration rate is shown as a percentage of the State 3 rate with each substrate. Results are for mitochondria oxidizing (**a**) succinate in the presence of nigericin, (**b**) succinate in the absence of nigericin, (**c**) glutamate with malate, or (**d**) pyruvate with malate. The flow ratio, $J_P/J_S$ (i.e. the effective P/O ratio as a fraction of $P/O_{max}$), is shown on the left side of each panel. The value of $J_P/J_S$ is independent of the value assumed for $P/O_{max}$. The effective P/O ratio, calculated as $J_P/J_S$ multiplied by the assumed value of $P/O_{max}$, is shown on the right side of each panel. Results were calculated from the fluxes obtained from Figure 1.

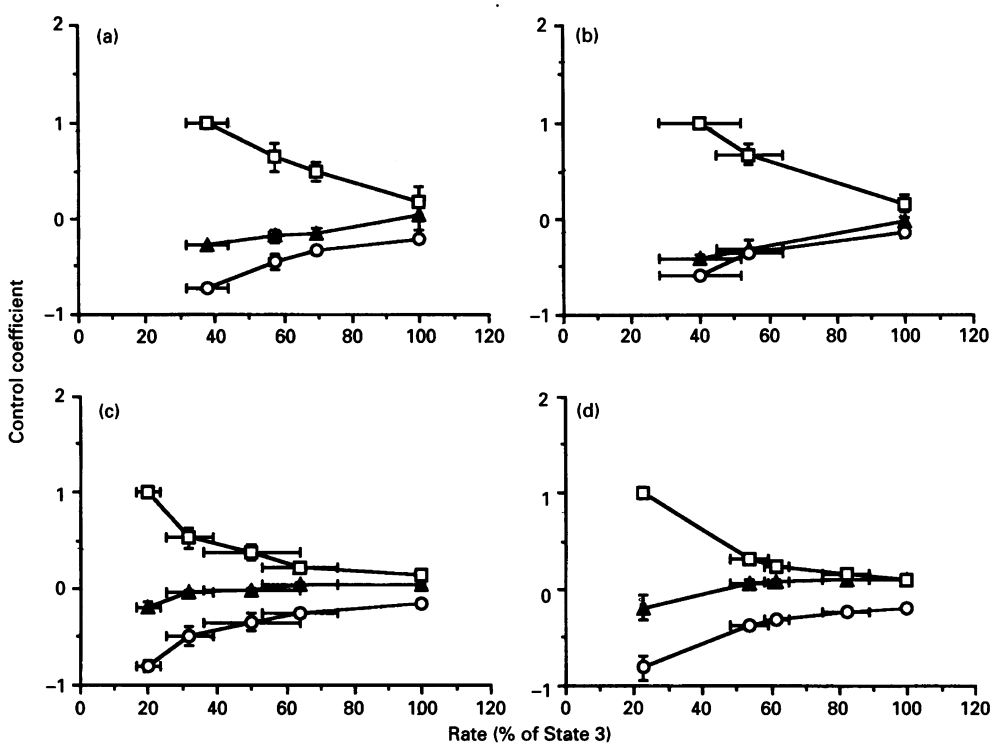


**Figure 7 Overall control coefficients of substrate oxidation, proton leak and the phosphorylation system over the effective P/O ratio in mitochondria**

Respiration rate is shown as a percentage of the State 3 rate with each substrate. Results are for mitochondria oxidizing (**a**) succinate in the presence of nigericin, (**b**) succinate in the absence of nigericin, (**c**) glutamate with malate, or (**d**) pyruvate with malate. Control coefficients were calculated from the flux control coefficients over respiration (Figure 2) and phosphorylation (Figure 3). Key: □, phosphorylation system; ○, proton leak; ▲, substrate oxidation.

**Table 1 Control coefficients over the effective P/O ratio in hepatocytes**

Results were calculated from the values of $J_P$, $J_S$, $C^{J_P}$ and $C^{J_S}$ given in *Brown et al. (1990b) and †Harper and Brand (1993). The flow ratio $J_P/J_S$ (i.e. the effective P/O ratio as a fraction of $P/O_{max}$) was calculated with $J_S$ representing either the total cellular oxygen consumption (A) or the mitochondrial oxygen consumption alone (B). The control coefficients over the effective P/O ratio were calculated as described in the Materials and methods section by using values for mitochondrial oxygen consumption.

| Rat hepatocytes... | Fed hypothyroid† | Starved euthyroid* | Fed euthyroid* | Fed euthyroid (hypothyroid control)† | Fed euthyroid (hyperthyroid control)† | Mean fed euthyroid |
|---|---|---|---|---|---|---|
| Flow ratio $J_P/J_S$ (effective P/O ratio as a fraction of $P/O_{max}$) | | | | | | |
| (A) $J_S$ = total oxygen consumption | 0.57 | 0.57 | 0.54 | 0.49 | 0.48 | 0.50 |
| (B) $J_S$ = mitochondrial oxygen consumption | 0.69 | 0.78 | 0.69 | 0.63 | 0.61 | 0.64 |
| Control over effective P/O ratio exerted by: | | | | | | |
| Substrate oxidation | 0.02 | −0.06 | −0.06 | 0.02 | 0.13 | 0.03 |
| Phosphorylation | 0.30 | 0.27 | 0.35 | 0.30 | 0.29 | 0.31 |
| Proton leak | −0.32 | −0.21 | −0.29 | −0.32 | −0.42 | −0.34 |

P/O ratio in hepatocytes. Like the control coefficients over the fluxes (Brown et al., 1990b; Harper and Brand, 1993), the values in resting hepatocytes are very similar to those in isolated mitochondria at intermediate respiration rates with each of the substrate systems examined. The mitochondrial proton leak had the greatest control coefficient over the effective P/O ratio in hepatocytes, with a value of −0.34. Thus any increase in the amount of proton leak would cause a substantial decrease in the effective P/O ratio. There was also strong control by the phosphorylation system, with a value of 0.31. Thus an increase in ATP demand, which would be equivalent to an increased activity of the block of reactions concerned with ATP synthesis and use, would cause a substantial increase in the P/O ratio, and would effectively recouple the mitochondria when ATP was required. There was much smaller control by the reactions of substrate oxidation, showing that increased substrate supply, for example, has little tendency to change the effective P/O ratio in resting hepatocytes.

## DISCUSSION

The control coefficients over the different branches of oxidative phosphorylation for each of the four substrate combinations investigated are very similar in general pattern and in most of the details. This suggests that the results for succinate are a valid picture of how the control is distributed for other substrates. The control coefficients obtained in the present work at 37 °C for the NAD-linked substrates are all very similar to those for succinate with or without nigericin and very similar to those obtained by Hafner et al. (1990) using succinate in the presence of nigericin at 25 °C. This suggests that the overall elasticities to $\Delta\psi$ of the different dehydrogenases and carriers involved under the different conditions are well matched, despite the known differences in their kinetics, although it should be noted that substrates were present at unphysiologically high, saturating, concentrations in the experiments with isolated mitochondia.

Studies of the control of respiration in heart and kidney mitochondria using different substrates report similar findings about flux control coefficients over respiration rate to those reported here for liver mitochondria. In heart mitochondria respiring in State 3 on succinate, pyruvate with malate or palmitoylcarnitine, the main control lies in the $\Delta p$ producers, especially the substrate dehydrogenases. Most of the control lies in the adenine nucleotide translocator (a component of the phosphorylating system) at intermediate rates of respiration and probably in the proton leak in State 4. We say 'probably' because the assay used to measure the control by the proton leak in this earlier work was invalid (see Brand et al., 1988), but tends to give values that are like those obtained by the valid assay used in the present paper. In heart mitochondria at intermediate states there is slightly more control in the phosphorylating system and probably less in the proton leak for NAD-linked substrates compared with succinate (Kholodenko et al., 1991). Heart and kidney mitochondria oxidizing pyruvate with malate or 2-oxoglutarate in State 3 with high substrate concentrations show only small differences in the sum of the control coefficients of the adenine nucleotide translocator, ATP synthase and the phosphate carrier (i.e. the phosphorylating system) over respiration for the two substrates (Moreno-Sánchez et al., 1991). As control by the proton leak is small in State 3, the control by substrate oxidation will also be fairly similar for the two substrates. However, as pointed out by those authors, the physiological concentrations of the NAD-linked substrates and of their effectors such as $[Ca^{2+}]$ are likely to be in the range where the dehydrogenases have greater control over respiration rate than they do at saturating substrate concentrations.

The variation of the effective P/O ratio with respiration rate between State 4 and State 3 in isolated mitochondria extends the earlier observations by Ernster and Nordenbrand (1974). The mitochondrial proton leak is a significant contributor to the rate of respiration in hepatocytes (Nobes et al., 1990a; Brown et al., 1990b; Harper and Brand), and the calculated effective P/O ratios in Table 1 show how the proton leak causes the effective P/O ratio in hepatocytes to be substantially less than the theoretical maximum value. As discussed previously (Brand, 1990), one of the functions of the proton leak in cells may be to allow the rate of ATP production to be relatively independent of the rate of proton pumping and oxygen consumption by the respiratory chain. This is shown by the high flux control coefficient of the phosphorylation system over the phosphorylation flux and the relatively low flux control coefficient of substrate oxidation over the phosphorylation system flux (Figure 3). Any changes in proton flux through the ATP synthase would be accompanied by changes in the proton leak rate and in the effective P/O ratio rather than by changes in the proton pumping rate, as shown by the high control over these variables by the phosphorylation system (Figures 4 and 7). There would be only small changes in the value of $\Delta\psi$, as shown by the small values of the concentration control coefficients over $\Delta\psi$ in Figure 5 and discussed by Duszynski et al. (1984).

The control coefficients over the effective P/O ratio have not been explicitly calculated previously. Our results show that the distribution of control is similar in isolated mitochondria and in hepatocytes. In each case the proton leak has negative control and ATP turnover has positive control; substrate oxidation has little control. Agents that affect the kinetics of the proton leak, such as thyroid hormones (Hafner et al., 1988) or phylogeny (Brand et al., 1991), will tend to alter effective P/O ratios with little effect on ATP production rate, although this depends on any other effects they also have. Straightforward stimulation of ATP demand will tend to decrease the contribution to respiration rate by the proton leak and increase the effective P/O ratio. Agents that stimulate the respiratory chain, such as glucagon (Brand et al., 1990) or added fatty acid substrates (Nobes et al., 1990b), will increase proton leak rate and respiration rate, but will also increase the rate of ATP synthesis, as shown by the low control coefficient of substrate oxidation over the effective P/O ratio in both mitochondria and cells, and so will have little effect on the effective P/O ratio, although other effects of each of these agents may again complicate the picture.

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