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Biochimica et Biophysica Acta 1118 (1994) 405–416

BBA Biochimica et Biophysica Acta

Characteristics of mitochondrial proton leak and control of oxidative phosphorylation in the major oxygen-consuming tissues of the rat

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Received 29 March 1994

Abstract

Maintenance of an electrochemical proton gradient across the mitochondrial inner membrane against the significant proton permeability of the membrane accounts for 25–30% of resting oxygen consumption in hepatocytes. It has been proposed that proton leak could be a significant contributor to resting metabolic rate in mammals if it were present in other tissues. Mitochondria were isolated from the major oxygen-consuming tissues (liver, kidney, brain and skeletal muscle) of the rat. In each tissue, the mitochondria showed significant proton leak with the same characteristic non-linear dependence on membrane potential. Liver and kidney mitochondria showed similar membrane proton permeability per mg of mitochondrial protein; brain and muscle permeabilities were greater when expressed in this way. Differences in the kinetic response of the substrate oxidation and phosphorylating systems to membrane potential were observed. The substrate oxidation system was more active in kidney, brain and skeletal muscle mitochondria than in liver mitochondria per mg of mitochondrial protein. Liver and kidney phosphorylating systems were less active than brain and skeletal muscle per mg of mitochondrial protein. The control of oxidative phosphorylation was also assessed. The distribution of control in mitochondria isolated from the four tissue types was found to be similar.

Keywords: Mitochondrion; Proton leak; Oxidative phosphorylation; Metabolic control

1. Introduction

Mammalian tissues will consume oxygen without doing any net work under certain defined conditions [1]. The observed metabolic rate of animals under those conditons is often termed standard, or resting metabolic rate. The reason that oxygen consumption occurs in the resting mammal (ignoring pressure/volume work by the heart and lungs, excretory work by the kidney etc.) is that the processes that accomplish internal work – production of low entropy structures such as proteins, lipid membranes and ion gradients – are not at equilibrium. This disequilibrium is due to pathways that uncouple metabolism and dissipate the low entropy structures, for example protein breakdown and the passive permeability of membranes to ions. Since no net work is accomplished, all resting oxygen consumption used to drive production of low entropy structures will appear as heat. Over the years, attempts have been made to identify these uncoupling reactions, and quantify their contribution to resting oxygen consumption, and therefore also to heat production. As well as being intrinsically interesting, such information might prove useful in identifying ways of improving the efficiency of food conversion in livestock and humans, and in indicating the significance of increased activity of uncoupling reactions in pathological states such as wasting diseases. Recent work indicates that major

Abbreviations used: $TPMP^+$, methyltriphenylphosphonium; FCCP, *p*-trifluoromethoxycarbonylcyanide-phenylhydrazone; $\Delta\Psi$, membrane potential; Δp, electrochemical proton gradient; ΔpH, pH gradient.

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SSDI 0005-2728(94)00145-6

contributors to total resting oxygen consumption include the Na^+/K^+ and Ca^{2+} ATPases (together accounting for around 20% of resting metabolic rate in adult rats, calculated from data in Refs. [2] and [3]) and protein turnover (around 20% in resting adult rats [4]). The contribution of the 'service' functions (kidney and heart work, respiration, nervous and liver functions) is estimated by Baldwin et al. [5] to be 36–50% of resting oxygen consumption. However, it is not clear to what extent the service functions are distinguishable from the other reactions mentioned (e.g., kidney and neuronal work is mainly ion transport) and hence the contribution of the service functions and ion transport to resting metabolic rate may not be additive.

An alternative way of explaining the origin of heat production in the basal state is to separate the 'internal' work done by the body from the reactions that produce the free energy to accomplish this work. It has been calculated that the 'internal' work done in the fasting, resting adult human accounts for only 10–15% of basal oxygen consumption [1]. Prusiner and Poe [6], by comparing the enthalpy of oxidation of glucose and fatty acids to that of ATP hydrolysis (assuming a value for the ATP/O ratio), calculated that 25% of cellular heat production is due to ATP-consuming reactions. It follows from these analyses that the reactions participating in the provision of ATP to accomplish the internal work are the major oxygen consumers in the resting state.

It is known that the bulk of cellular oxygen consumption (85–90% [7,8]) in resting, isolated liver cells is mitochondrial. In other organs this figure is probably even higher due to the considerably higher activities of non-mitochondrial oxygen consuming protein [9] in liver. Mitochondria use the free energy derived from the oxidation of food molecules to produce an electrochemical proton gradient (Δp) across their inner membrane. The passive permeability of this membrane to protons ('proton leak') is significant – in isolated rat liver cells [7,8] and thymocytes [10] oxygen consumption to maintain Δp against the proton leak accounts for around 20–30% of the total resting respiration rate. It is therefore possible, as proposed by Brand [11], that the maintenance of a Δp against the proton leak could account for a significant proportion of the total resting oxygen consumption of an organism.

The present paper confirms the presence of the proton leak in the major oxygen-consuming tissues of the rat and investigates its properties. This is a necessary first step in an assessment, using intact tissues, of the contribution of proton leak to resting oxygen consumption in the rat. Control of oxidative phosphorylation in these tissues is also assessed, although it should be noted that this is not related to the question of the importance of proton leak as a contributor to Standard Metabolic Rate.

2. Experimental

2.1. Methods

Preparation of mitochondria

Unless otherwise stated, all mitochondria were prepared from 2- to 3-month-old female Wistar rats within the weight range 220–260 g. They were maintained at a thermoneutral temperature, provided with access ad lib. to a complete diet (Rat and Mouse No. 1 [modified]; SDS, 1 Stepfield, Witham, Essex) and drinking water and were killed by stunning followed by decapitation. Mitochondrial protein was assayed by the Biuret method [12], using bovine serum albumin (BSA) as a standard. Mitochondrial protein:lipid ratios were determined by extracting lipid from a known quantity (assayed as mg of mitochondrial protein) of isolated mitochondria as described by Else and Hulbert [13]. Homogeniser total clearences were as follows: tight fit, 0.18 mm; medium fit, 0.48 mm; loose fit, 0.60 mm.

(a) *Liver.* Liver mitochondria were prepared by the method of Chappell and Hansford [14] in a medium containing 250 mM sucrose, 5 mM Tris and 1 mM EGTA, adjusted to pH 7.4 with HCl.

(b) *Kidney.* Kidney mitochondra were prepared by the method of Johnson and Lardy [15] with some modifications. The entire kidney was homogenised (after first removing the capsule) using 7 strokes with a medium fit pestle and centrifuged at $1050 \times g$ for 3 min. The supernatant was retained, the pellet rehomogenised using 4 strokes with a tight fit pestle and the homogenate was centrifuged as before. In initial preparations the supernatants from the different homogenisation steps were kept separate, and the respiratory characteristics of the mitochondria derived from each was determined separately. However, these characteristics were unaffected by the different regimes and hence in later preparations the supernatants were combined after the second centrifugation step. The combined supernatants were then treated in the same way as the liver preparation. The isolation medium was the same as that used for the liver except that 1% (w/v) defatted BSA was included.

(c) *Brain.* Mitochondria were isolated from whole brains by 'method A' of Sims [16] with minor modifications. Rats were killed by decapitation without stunning and the brain was removed to buffer on ice within 1 min. The initial homogenisation (5 brains at a time) used 7 strokes with the medium fit pestle. Following centrifugation ($1300 \times g$; 3 min) the pellet was rehomogenised using 4 strokes with the tight fit pestle before being recentrifuged ($1300 \times g$; 3 min). The effects of the different homogenisation regimes on the respiratory characteristics of the mitochondria was checked as for the kidney and found to be negligible. The supernatants from the previous centrifugation steps

were pooled, and spun at 21000 × *g* for 10 min. The pellet was resuspended in 15% Percoll made up in isolation medium, layered onto a gradient as described by Sims [16], and spun at 30500 × *g* for 5 min. The material at the interface of the lower two percoll layers was removed by pipette, diluted 1:4 by gently mixing with isolation buffer plus 1% (w/v) BSA and spun at 16000 × *g* for 10 min. As much supernatant as possible was removed without disturbing the resultant fluffy pellet, the pellet was diluted 10-fold with isolation medium and spun at 6000 × *g* for 10 min. The pellet was gently resuspended and stored on ice. The isolation medium contained 320 mM sucrose, 10 mM Tris and 1 mM EGTA, adjusted to pH 7.4 with HCl.

(d) *Skeletal muscle.* Mitochondria were isolated from abdominal and hind leg muscle by the method of Bhattacharya et al. [17] with minor modifications. 20–30 g of hindquarter muscle (including all leg muscles and a small amount of back muscle) was removed to ice-cold isolation medium (100 mM sucrose; 9 mM EDTA; 1 mM EGTA; 100 mM Tris HCl; 46 mM KCl; pH 7.4). Muscle was finely chopped on a precooled porcelain tile with a razor blade, then added to 300 ml of isolation medium containing 0.5% (w/v) BSA and 20 mg% (w/v) Nagarse (Type XXVII; 7.8 units/mg) and incubated on ice, with stirring, for 5 min. The tissue was then further disrupted using an MSE 'overhead' food mixer (half maximum speed; 15 s) and then spun down at 1300 × *g* for 3 min. The supernatant was spun at 12000 × *g* for 10 min, and any white fluffy material on top of the pellet was removed by gentle swirling. The pellet was then resuspended in 10 ml of isolation medium and respun at 12000 × *g* for 10 min. This regime was repeated two or three times, until no fluffy white material was spun down with the pellet. The pellet was then resuspended in ice cold isolation medium and stored on ice. The enzyme-digest/homogenisation regime was varied (from just homogenising for various times through several permutations of enzyme digest + homogenising to just enzyme digest for various times) in an attempt to improve the quality of the isolated mitochondria. The regime outlined above was the one that produced the preparation with the highest Respiratory Control ratio [18].

Titrations of membrane potential

Measurements of oxygen consumption and membrane potential were carried out simultaneously in the same vessel. Membrane potential was determined by using a $TPMP^+$-sensitive electrode as described by Brown and Brand [19]. Mitochondria (0.5 mg protein/ml – liver and kidney; 0.25 mg/ml – brain and skeletal muscle) were incubated in a thermostatically controlled oxygen electrode chamber at 37°C in 3.5 ml 100 mM KCl, 20 mM sucrose, 20 mM glucose, 10 mM KH_2PO_4, 5 mM Hepes, 2 mM $MgCl_2$ and 1 mM EGTA (pH 7.2 with KOH), plus 5 mM (K^+) succinate, 0.4 μg nigericin/mg mitochondrial protein and 5 μM rotenone. Titrations of state 4 respiration were carried out either in the presence of 2 μg oligomycin/mg mitochondrial protein by sequential additions of 1 M K^+-malonate (to give final concentrations of 0.33, 0.67, 1, 2, 3, 5 and 10 mM) or in the absence of oligomycin with 100 μM ATP and small aliquots of hexokinase. Titrations of state 3 respiration were carried out, in the presence of 100 μM ADP and sufficient hexokinase (0.65 units/ml) to maintain state 3, by sequential addition of 1 M K^+-malonate to give final concentrations of 0.33, 0.67, 1.0 and 2.0 mM. At the beginning of the trace the TPMP electrode was calibrated by sequential (1 μM) additions of TPMP-bromide over the range 1–5 μM in the presence of mitochondria. At the end of each trace the point on the trace where $\Delta\Psi$ was zero was determined by addition of a final concentration of 20 mM malonate and 200 pmol FCCP/mg mitochondrial protein. This enabled 'drift', in the system to be estimated. The drift was never more than ±10% of the original full scale chart recorder deflection. The electrode measured the external $TPMP^+$ concentration. The internal amount of $TPMP^+$ was calculated from the amount of $TPMP^+$ added minus the amount remaining externally. Membrane potentials were calculated using the Nernst relationship and the appropriate correction for binding of the probe (see Table 1), assessed for mitochondria from each tissue using the method of Brown and Brand [20]. Note that the value of the mitochondrial $TPMP^+$ binding correction (Table 1) is determined as a function of the matrix volume and has units of mg of mitochondrial protein per μl of matrix volume. Thus, when the amount of $TPMP^+$ taken up by the mitochondria (measured per mg of mitochondrial protein) is multiplied by the $TPMP^+$ binding correction (mg/μl), the resulting number is the free matrix $TPMP^+$ concentration.

The concentrations of the various reagents used for the liver experiments were as used by previous workers [21,22], but were checked when used with mitochondria from different tissues to ensure sufficient was being added for the full effect. The reagents tested in this way were FCCP, nigericin, oligomycin, ADP, succinate and rotenone. The concentrations of these compounds used with liver mitochondria were found to be sufficient for full effect in the other tissues tested.

Measurement of oxygen consumption

Respiration rates were measured in the same experiment as membrane potential using a Clarke-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) in the base of the incubation vessel. Oxygen consumption rates were calculated assuming that the

concentration of oxygen in the air-saturated incubation medium was 406 nmol O/ml at 37°C [23].

Fatty acid assay

The esterified fatty acid composition of mitochondrial phospholipids from each organ was determined by a modified method of Hulbert and Else [24]. Basically, total lipids were extracted from mitochondria – prepared as described previously – using chloroform/methanol (2:1, v/v) in the presence of antioxidant (0.01% (w/v) butylated hydroxytoluene). Phospholipids were separated by silicic acid column chromatography. Phospholipid fatty acids were methylated using 14% BF_3 in methanol. Methyl esters of the fatty acids were extracted with petroleum spirit, purified by hydrated florisil (florisil + 7% water) column chromatography and separated on a Varian 3300 gas chromatograph with a capillary column (S.G.E. column type 25QC2/BPX70) and a flame ionisation detector. Column temperature was initially 80°C and was raised (15 C°/min) to 150°C, at which level it was maintained. Individual fatty acid methyl esters were identified by comparison with known standards. Relative composition (weight %) was determined with a Shimadzu C-R3A chromatopac integrator.

2.2. Materials

All materials were obtained from Sigma Chemical Co., Fancy Road, Poole, UK, except for $TPMP^+$-bromide (Aldrich Chemical Co., Gillingham, UK), adenine nucleotides (Boehringer Mannheim (UK), Lewes, UK).

3. Results and discussion

3.1. Mitochondrial preparations

The major problem in attempting to compare the proton leak in mitochondria isolated from different tissues is the relative resistance to disruption of the tissues themselves. Clearly, the more force required to disrupt an organ, the more damaged the mitochondria are likely to be. For this reason, the isolation method was chosen carefully, the main criteria for this choice being (a) experience of the authors in this field, (b) compatibility of the aims of the method with those of this project – e.g., a method for isolating mitochondria for subsequent protein purification would probably not produce a suitably coupled organelle for the study of oxidative phosphorylation, (c) the number of times the method has been cited by other researchers in a similar field (presumably an index of how successful and/or easy to use the method is, bearing in mind how recently published the method was) and (d) the reported characteristics of the isolated mitochondria.

Regarding this last criterion, one indicator of the damage done to mitochondria during their isolation is the Respiratory Control ratio (RCR). As defined by Chance and Williams [18] this is the state 3 respiration rate divided by the state 4 rate. A high state 4 rate, possibly indicating damage to the inner membrane, or a low state 3 rate would lead to a reduced RCR. However, the RCR is a crude assay of mitochondrial damage for many reasons. For example, it is clear that the RCR depends not only on the permeability of the inner membrane but also on the activity of the respiratory chain and ADP phosphorylating enzymes. The balance of these three factors may be different in mitochondria from different tissues and hence an inter-tissue comparison of mitochondrial RCR is not necessarily an index of differential isolation damage. Furthermore, the RCR may depend on the medium used. Brain mitochondria in a sucrose-based medium have a RCR of around 4.0, compared to a value of 2.6 for the same preparation in a KCl-based medium such as the one given in the experimental section. Hence, the RCR is only really useful as an rough index of mitochondrial integrity, and then only if it has been assessed under the same conditions and is used to compare organelles from the same tissue.

The RCR of our own preparations (Table 1) generally compared favourably with those of the authors of the isolation methods (or by other workers using the same methods) used in this study. Hafner and coworkers [21] obtained a RCR of around 4.2 for rat liver mitochondria, similar to our figure of 3.9. These values are lower than those obtained with the same preparations in the absence of nigericin. Gmaj et al. [25], using rat kidney cortex mitochondria, obtained a RCR of 2.5 ± 0.4, although our value of 2.9 ± 0.6 was for mitochondria from whole kidney. Also the RCR of our brain preparations, 2.9 ± 0.7, is the same as that (3.0 ± 0.1) obtained by Sims [16]. However, the result for muscle (2.0 ± 0.2) does not compare favourably with that of Bhattacharya et al. [17]: 4.5 ± 0.7. This result was not due to the difference in the incubation media used to carry out respiration measurements (we used a KCl-based medium, whereas Bhattacharya employed a mannitol/sucrose medium). We tested this using our mitochondria in the mannitol/sucrose medium of Bhattacharya and obtained no significant improvement in RCR. If the low RCR was due to inner membrane damage, one might expect its permeability to externally added NADH to be significant. The effect on muscle mitochondrial state 3 respiration (where substrate supply has the greatest control over respiration rate) of externally adding 100 μM NADH was compared with the state 3 rate in the presence of glutamate (2.7 mM) and malate (0.7 mM). The increase in respiration due to addition of external NADH was 12–15% of the increase obtained with glutamate and malate. Thus the

membrane did not seem to be unduly damaged, although this method would tend to underestimate such damage, as the driving force ($\Delta\Psi$; not measured) for the phosphorylation rate in the presence of external NADH would have been much lower than that with glutamate and malate. If the low RCR was due to loss of chain protein (e.g., cytochrome *c*) either during isolation, or during incubation at physiological ionic strength and temperature (i.e., if the state 3 rate is limited by the reduced activity of the chain), one might expect the respiration rate in the presence of FCCP to be similar to the state 3 rate. We obtained an FCCP-stimulated respiration rate of 784 ± 65 nmol O/min per mg compared with the state 3 rate of 435 ± 60. Note that we also obtained a similar FCCP effect in mitochondria from liver, brain and kidney. Similarly, if the chain was damaged, one would expect to observe a low membrane potential, whereas the potential in muscle mitochondria was similar to that of liver mitochondria. Furthermore, loss of cytochrome *c* is probably not occuring due to the 37°C incubation temperature used in this study. Incubation of skeletal muscle mitochondria at either 37°C or 30°C in otherwise identical conditions had no significant effect on RCR (data not shown). Hence, using these criteria, the chain activity does not appear to be significantly impaired. The use of nigericin (not used by Bhattacharya) affects RCR. We found that in the absence of nigericin (0.4 μg/mg) the RCR of muscle mitochondria was 2.7 ± 0.1. Nevertheless, it is possible that our muscle mitochondria have higher state 4 respiration rates because they have been more damaged by the isolation procedure than the other mitochondria, which are derived from much softer tissue.

In general, then, the mitochondria used in this study compared favourably with the best prepared by other workers. They are therefore considered to be representative enough, with the possible exception of those from skeletal muscle, to be used as approximate guides to the qualitative characteristics of such organelles, although clearly no quantitative statements can be made until the work is repeated in cells or whole organs.

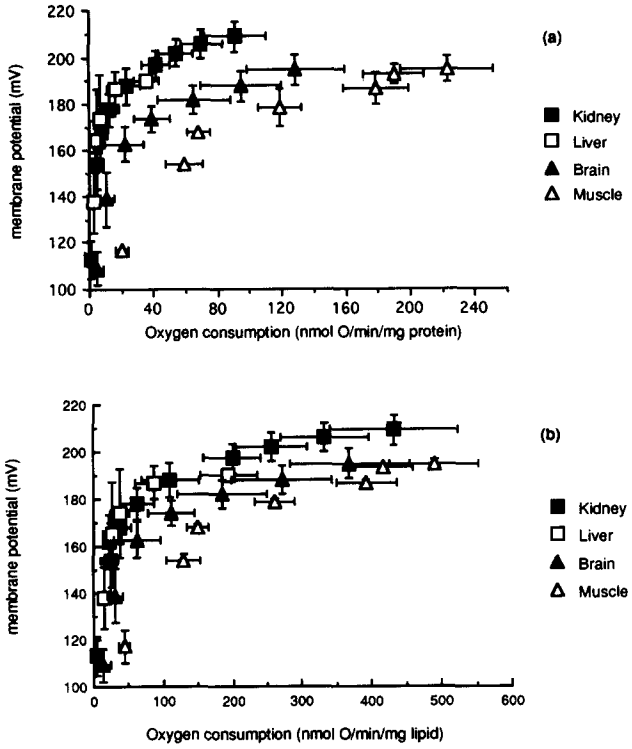

Fig. 1. The kinetic response of the proton leak to membrane potential in mitochondria isolated from the major oxygen-consuming organs of the rat. Conditions of isolation and assay were as described in the Experimental section. Data represent titrations of state 4 respiration in the presence of oligomycin (2 μg/mg protein) by sequential additions of potassium malonate. (a) Oxygen consumption data expressed per mg of mitochondrial protein and (b) the same data corrected for inter-tissue differences in mitochondrial protein to lipid ratios (see text for explanation). Data are expressed as the mean ± S.E. of three independent experiments.

Table 1
Characteristics of mitochondrial preparations

Organ	Liver	Kidney	Brain	Skeletal muscle
Yield (mg protein/g tissue)	8	7	1–2	1–2
Oxygen-consumption rate: (nmol O/min per mg protein)				
(a) State 3	137 ± 17 (3)	252 ± 77 (3)	362 ± 93 (3)	435 ± 60 (3)
(b) State 4	35 ± 6 (3)	87 ± 23 (3)	125 ± 25 (3)	222 ± 27 (3)
Respiratory Control Ratio	3.9 ± 0.6 (3)	2.9 ± 0.6 (3)	2.9 ± 0.7 (3)	2.0 ± 0.2 (3)
$TPMP^+$ binding correction $(\mu l/mg\ protein)^{-1}$	0.42 ± 0.09 (3)	0.51 ± 0.23 (3)	0.30 ± 0.05 (3)	0.35 ± 0.10 (3)
Lipid:protein ratio (mg/mg)	0.19 ± 0.01 (2)	0.21 ± 0.02 (2)	0.35 ± 0.00 (2)	0.46 ± 0.02 (2)

All rates were obtained using succinate (plus 5 μM rotenone) as the respiratory substrate in the presence of nigericin (0.4 μg/mg protein) at 37°C in KCl-based medium as described in the Methods section. 'State 3', 'state 4' and 'Respiratory Control ratio' were as defined by Chance and Williams [18]. TPMP binding correction was assessed using the method of Brown and Brand [20]. Lipid:protein ratios were determined as described by Else and Hulbert [13]. Values shown are the means ± S.E. (number of independent preparations tested), except lipid:protein ratios, which are shown as mean ± range of two independent preparations.

3.2. Kinetics of the proton leak

Fig. 1 shows the kinetic response of the proton leak to $\Delta\Psi$ in mitochondria isolated from liver, kidney, brain and skeletal muscle. Note that throughout this discussion we will assume that the H + /O stoichiometry of the respiratory chain is invariant, a position for which ample evidence exists [26–29]. State 4 succinate respiration (in the presence of oligomycin) was titrated with an inhibitor of succinate dehydrogenase, and $\Delta\Psi$ and oxygen consumption were measured simultaneously. Fig. 1a shows the response to $\Delta\Psi$ of the oxygen consumption used to drive the proton leak flux, when measured as nmol of oxygen consumed/min per mg mitochondrial protein as described previously [21,30, 31]. It shows that proton leak is present in all the tissues analysed and that it exhibits the characteristic non-linear dependence on $\Delta\Psi$ first observed by Nicholls in rat liver mitochondria [32]. The question that arises from Fig. 1a is "does the observed difference in leak kinetics reflect an increased proton permeability of the inner membrane or just an increased surface area of inner membrane per mg protein for protons to leak back across?" In an attempt to answer this question we have also calculated the leak oxygen consumption per mg mitochondrial lipid (Fig. 1b) using mitochondrial mg protein:mg lipid ratios (see Table 1) obtained by extracting the lipids of isolated mitochondria as described in the Methods section. The lipid: protein ratios determined by us show the same trend as those measured by Hulbert and Else [24] in rat. The use of lipid:protein ratios to assess the inner mitochondrial membrane surface area:protein ratio assumes that all the lipid and protein in the isolated mitochondrial fraction is mitochondrial, and that total mitochondrial lipid is a valid measure of inner membrane surface area for comparative purposes. Total lipid measurements will include the outer mitochondrial membrane, which from the stereological data used by Else and Hulbert [33] we have calculated to approximate 24%, 13%, 18% and 19% of total mitochondrial phospholipid content (assuming mg phospholipid per unit membrane surface area is the same for inner and outer mitochondrial membranes) for liver, kidney, brain and skeletal muscle, respectively. From these data it appears that the values for mitochondrial inner membrane surface area calculated using total lipid:mg protein ratios are underestimated in all mitochondria by a similar amount. The assumption that all of the protein measured by the Biuret assay in the isolated mitochondrial preparations is mitochondrial is least tenable for muscle mitochondria. Preparations of these organelles may have salt-soluble actinomyosin contamination and hence may have their calculated state 4 oxygen consumption/mg lipid values overestimated by this method. However, the results of Mickelson et al. [34] show that removal of non-mitochondrial protein contamination by Percoll gradient centrifugation has no significant effect on the respiratory parameters of bovine skeletal muscle mitochondria, measured as oxygen consumption per mg mitochondrial protein. Thus, perhaps non-mitochondrial protein contamination of our rat muscle mitochondrial preparations is insignificant.

It can be seen from Fig. 1b that the proton permeabilities of liver and kidney mitochondrial inner membranes are the same at any given membrane potential, and that a difference in mg lipid:mg protein ratio (Table 1) accounts for around half of the difference in proton leak kinetics between liver and kidney and brain and muscle shown in Fig. 1a. Fig. 1b also shows that the increased state 4 respiration rate observed in kidney mitochondria is due to maintenance of a higher membrane potential – the result of a more active electron transport chain, due either to a difference in the kinetics of the kidney chain proteins or to an increase in the mg chain protein:mg total protein ratio (see Fig. 2a). It is interesting to note that the increased capacity of the chain to pump protons is not matched

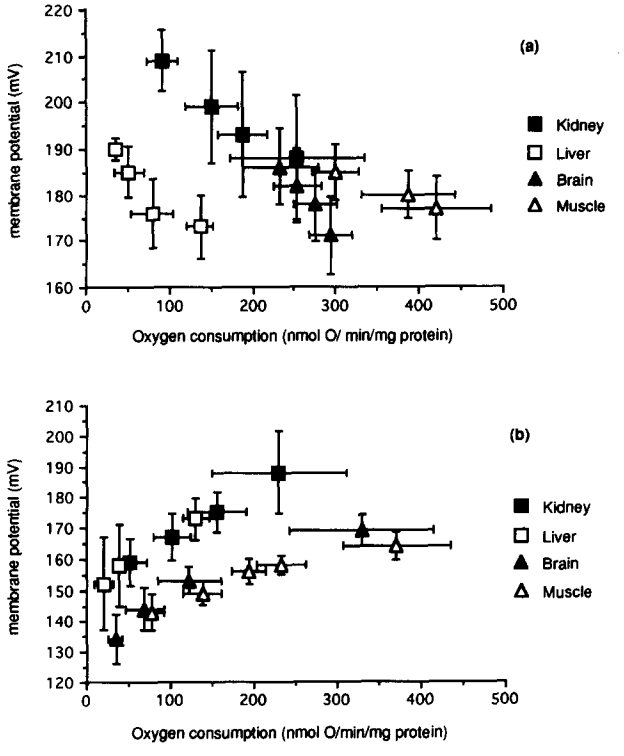

Fig. 2. The kinetic response of (a) the substrate oxidation system and (b) the phosphorylating system to membrane potential in mitochondria isolated from various organs of the rat. Conditions of isolation and assay were as described in the Experimental section. In Fig. 2b the oxygen consumption driving phosphorylation at any given value of $\Delta\Psi$ was calculated as {total oxygen consumption − oxygen consumption at that value of $\Delta\Psi$ in the presence of oligomycin}. Data are expressed as the mean ± S.E. of three independent experiments.

by an increase in phosphorylating system activity, as evidenced by the lower RCR of kidney mitochondria compared to liver (Table 1 and see also Fig. 2b). One consequence of this is that the steady state $\Delta\Psi$ at any given phosphorylating flux will be higher than in liver and thus a greater proportion of total mitochondrial respiration would be required to drive the leak in kidney mitochondria, especially at high $\Delta\Psi$. The quantitative significance of this in vivo would depend on the value of the resting $\Delta\Psi$ in intact kidney.

Finally, it is possible that some of the differences in leak kinetics seen in Fig. 1b may be due to uncoupling by free fatty acids [35]. An interesting question regarding free-fatty-acid-induced proton leak is whether or not it is an artefact of mitochondrial isolation. Assuming that it is (which is by no means clear) this would only affect our (tentative) conclusion that the inner membrane proton permeability is different in the different mitochondrial types if the degree of fatty acid uncoupling was different in these mitochondria. This is clearly a possibility and was the reason for including BSA (which adsorbs free fatty acids) in the isolation media. However, we consider that the effect of any uncoupling due to free fatty acids would not alter the main conclusions of the paper – that proton leak is present and has significant activity in mitochondria from the major oxygen-consuming tissues – since, in liver mitochondria at least, the proportion of the proton leak that can be inhibited by BSA is only about 23% of state 4 respiration [35].

3.3. Esterified fatty acid composition of mitochondrial membrane lipids

An increase in total lipid to protein ratio accounts for around half of the difference between the leak kinetics of liver and kidney mitochondria compared with skeletal muscle and brain mitochondria (Fig. 1a and b). Thus, it appears that the remaining difference is due to an increased proton permeability of the brain and muscle mitochondrial inner membranes. We analysed the esterified fatty acid composition of mitochondrial membrane lipids from liver, brain, kidney and skeletal muscle in an attempt to examine whether the greater leakiness of brain and muscle mitochondrial membranes is due to a change in the fatty acid composition of the membranes (Table 2). Altered mitochondrial proton permeability in lizards compared to rats

Table 2
Fatty acid composition of phospholipids of mitochondria isolated from rat liver, kidney, skeletal muscle and brain

Fatty acid	Origin of mitochondria			
	Liver	Kidney	Skeletal muscle	Brain
16:0	11.3 ± 1.7	12.5 ± 0.2	12.7 ± 1.1	14.0 ± 0.3
16:1($n-7$)	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	1.1 ± 0.0
17:0	0.5 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
17:1		0.5 ± 0.0	0.4 ± 0.1	1.1 ± 0.0
18:0	22.7 ± 2.4	13.1 ± 0.1	10.9 ± 0.5	14.0 ± 0.1
18:1($n-9$)	3.4 ± 0.5	6.6 ± 0.1	3.4 ± 0.0	12.9 ± 0.0
18:1($n-7$)	1.7 ± 0.1	3.2 ± 0.0	2.3 ± 0.1	3.7 ± 0.1
18:2($n-6$)	13.6 ± 1.1	19.3 ± 0.4	21.8 ± 0.7	2.1 ± 0.1
18:3($n-3$)		0.1 ± 0.1	0.1 ± 0.1	
20:3($n-9$)	0.4 ± 0.1	0.4 ± 0.0	0.2 ± 0.1	0.1 ± 0.1
20:3($n-6$)	1.8 ± 0.1	1.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.0
20:4($n-6$)	20.3 ± 0.6	28.0 ± 0.0	10.4 ± 0.2	17.2 ± 0.4
20:5($n-3$)	1.6 ± 0.5	1.1 ± 0.1	0.3 ± 0.3	
22:1($n-9$)	0.2 ± 0.4	0.1 ± 0.0		
22:4($n-6$)	0.1 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	1.8 ± 0.0
22:5($n-6$)	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.1	0.7 ± 0.4
22:5($n-3$)	1.2 ± 0.2	0.3 ± 0.0	1.8 ± 0.1	0.2 ± 0.0
22:6($n-3$)	13.2 ± 2.1	3.6 ± 0.0	26.5 ± 3.1	22.7 ± 0.1
Unsaturation index	230.6 ± 14.8	214.7 ± 0.7	286.7 ± 16.3	261.2 ± 1.1
Average chain length	19.0 ± 0.0	18.6 ± 0.0	19.2 ± 0.1	19.2 ± 0.1
% total unsaturates	64.9 ± 0.2	71.6 ± 0.3	74.3 ± 2.0	69.7 ± 0.4
% monounsaturates	5.8 ± 0.1	12.2 ± 0.2	7.5 ± 0.1	20.6 ± 0.1
% polyunsaturates	56.1 ± 3.5	59.4 ± 0.4	66.9 ± 2.0	49.1 ± 0.3
% ($n-6$) polyunsaturates	39.5 ± 0.2	53.4 ± 0.3	35.7 ± 1.1	24.2 ± 0.1
% ($n-3$) polyunsaturates	19.4 ± 0.4	5.6 ± 0.1	31.0 ± 4.0	24.7 ± 0.2

Values are mole percentages of total fatty acids expressed as means ± S.E. for liver mitochondria[a] ($n = 7$) or means ± range for kidney, muscle and brain ($n = 2$); n = the number of individual fatty acid assays carried out. The number of animals used in each assay was 1 (liver), 2 (kidney), 2 (muscle) and 15 (brain). Fatty acids are designated by the number of carbon atoms:number of double bonds (position of most distal double bond). The unsaturation index is the average number of double bonds per 100 fatty acids.

[a] Figures for liver include data kindly supplied by Dr R.K. Porter, compiled under identical conditions to those of our own study.

[22] and in hypothyroid compared to euthyroid rats [36] has previously been correlated with different fatty acid composition of the mitochondrial membrane bilayer.

It has been proposed [37] that esterified linoleic acid (18:2) acts as a 'proton plug' in mitochondrial membranes. However, as can be seen from Table 2, differences in the 18:2 content do not correlate with the observed differences in mitochondrial proton leak kinetics (Fig. 1b). Brand et al. [22] correlated the presence of the highly polyunsaturated fatty acids arachidonic acid (20:4) and docosahexaenoic acid (22:6) in rat liver mitochondria and their absence or reduced presence in lizard liver mitochondria with the much greater proton leak in the mammalian mitochondria. Table 2 shows that the mitochondria with the highest apparent proton permeabilities (brain and muscle) also have the highest mole percentage of 22:6, although the converse is true for 20:4.

It has been shown [38] that changes in the amount of $n-6$ unsaturated fatty acids in membrane lipids disrupts the packing of the fatty acyl chains at the centre of the lipid bilayer. According to the Eyring model of the ion permeability barrier in insulating membranes [39], the point in the bilayer at which the permeability barrier is greatest is its centre. Thus, it might be supposed that packing disruption at the centre of the bilayer would result in a more significant change to its permeability characteristics than similar disruptions elsewhere in the membrane. Since $n-3$ polyunsaturates have double bonds more terminal (i.e., towards the methyl end of the fatty acid molecule) than $n-6$ polyunsaturates, they are likely to be even more disruptive to packing in the middle of the bilayer and thus also to be more disruptive to the ion barrier properties of membranes.

In this light, it is of interest that the mitochondria with the greatest proton permeabilities had the most $n-3$ polyunsaturated fatty acid (PUFA) present in their membranes. However, there is not a simple correlation between $n-3$ PUFA content and proton leak in liver and kidney mitochondria. The relatively similar leak in mitochondria from these organs may be due to a different balance between $n-3$ and $n-6$ fatty acids.

Finally, the overall degree of unsaturation of membrane lipids, as shown by the unsaturation index, has previously been correlated with changes in proton permeability [22] – the higher the unsaturation index, the higher the leak. Table 2 shows that the mitochondria with the higher proton permeabilities also have the higher unsaturation indices.

We did not separate the inner and outer membranes of the mitochondrial samples before analysis and hence any observed trend in the differences in inner membrane composition may have been affected by the presence of outer membrane lipids – the outer membrane represents a significant proportion (around 25%, [40]) of the total membrane surface area in liver mitochondria. As mentioned previously, this proportion is apparently similar in mitochondria of kidney, brain and muscle. In liver, there are differences in fatty acid composition between the inner and outer membranes, most notably in 18:1 and 18:2 content [41]. However, the value obtained (expressed as area percent) for 18:1 and 18:2 (and also 20:4 and 22:6) content of the inner membrane is very similar to that obtained when the same analysis is done using whole mitochondria [41]. Hence the fatty acid composition of whole mitochondria seems, in liver at least, to accurately reflect the composition of the inner membrane.

The purity of the mitochondrial preparations used in the fatty acid analyses was assumed (but not proved) to be similar for each organ. If, say, brain mitochondria were more contaminated with non-mitochondrial lipid than the preparations from other organs, this might affect the observed differences in mitochondrial fatty acid content. However, it appears that the phospholipid fatty acid profile of whole liver [42] and heart [43] is similar to that of mitochondria isolated from the same tissue. Therefore non-mitochondrial lipid contamination would probably not significantly affect the observed mitochondrial membrane esterified fatty acid patterns.

All of the above conclusions assume that the measure of mitochondrial inner membrane surface area (Table 1) used to produce Fig. 1b is valid. However, using the stereological data of Hulbert and Else ([24] and unpublished data), the calculated values for mitochondrial inner membrane surface area per mg protein for rat liver, kidney, brain and muscle do not show the same pattern as the mg total lipid:mg protein ratios measured by us (Table 1) and Hulbert and Else [24]. Thus, any conclusions we might make based on the latter data are considerably weakened.

3.4. Kinetics of the substrate oxidation and phosphorylating systems

Fig. 2a shows the response of the substrate oxidation system to $\Delta\Psi$. Mitochondria in the standard incubation medium plus ATP (100 μM) and in the absence of oligomycin were titrated by addition of small aliquots of hexokinase solution. This method was used to manipulate $\Delta\Psi$ by making small changes to the activity of the phosphorylating system. This enabled the response of the substrate oxidation system to $\Delta\Psi$ to be assessed as previously described [21,30,31]. Fig. 2a expresses the activity of the substrate oxidation system at any value of $\Delta\Psi$ as the rate of oxygen consumption per mg mitochondrial protein. It shows that the liver substrate oxidation system is less active than that of mitochondria from the other organs, since it consumes

oxygen more slowly at any given value of $\Delta\Psi$. This would explain why isolated kidney mitochondria maintain a higher $\Delta\Psi$ than liver mitochondria; it would also account for the ability of brain and muscle mitochondria to maintain the same $\Delta\Psi$ as liver at higher leak rates.

Fig. 2b shows the response of the phosphorylating system to $\Delta\Psi$. This data set was obtained by titrating mitochondrial state 3 succinate respiration with an inhibitor of the respiratory chain (malonate). This enabled $\Delta\Psi$ to be manipulated. The response to $\Delta\Psi$ of the oxygen consumption being used to drive the phosphorylating system was determined by subtracting, at any value of $\Delta\Psi$, the oxygen consumption due to proton leak from the total oxygen consumption as previously described [8,21]. This is only valid as an indirect measurement of mitochondrial ATP synthesis if the H^+/O stoichiometry of the respiratory chain and the H^+/ATP ratio of the phosphorylating system remain constant as $\Delta\Psi$ is varied. There is ample evidence to support this position [26,27,28,29] and contrary evidence [44,45] is now considered [26,28] to be in doubt. Thus we consider that the data in Fig. 2b represent a suitable assay of the activity of the phosphorylating system at any given value of $\Delta\Psi$. Fig. 2b shows that brain and skeletal muscle mitochondria have a more active phosphorylating system (i.e., have a faster rate of ATP synthesis and turnover at any given value of $\Delta\Psi$) than that of the liver and kidney. This, together with the rest of the kinetic data, explains why the kidney has such a low Respiratory Control ratio whilst maintaining a high $\Delta\Psi$. The activity of the kidney substrate oxidation system is higher than in liver and so the $\Delta\Psi$ and the proton leak are high. However, the activity of the kidney phosphorylating system is the same as the liver, and hence the increased state 4 rate due to stimulation of the leak is not matched by a proportional increase in the maximal phosphorylating (state 3) rate.

The use of nigericin in our experiments may have some effect on the mitochondrial properties outlined in this paper. For example, we have noted that nigericin lowers the Respiratory Control Ratio (RCR) in brain and skeletal muscle mitochondria, although it has no effect on the RCR of those from liver. It is possible, therefore, that nigericin uncouples certain types of mitochondrion. However, we measured the change in the membrane potential of mitochondria isolated from different tissues on addition of saturating amounts of nigericin. We found that this change was around 10–20 mV in all cases (data not shown). If nigericin contributed to a significant uncoupling of mitochondria, one might expect to see a much smaller increase, or even a decrease in membrane potential.

Furthermore, it appears that nigericin does not stimulate the proton leak to a different extent in mitochondria from different tissues. In liver mitochondria, nigericin stimulates the state 4 rate by around 40% [46]. We have found that nigericin stimulates state 4 respiration in brain mitochondria by 32% and in muscle mitochondria by 40% (data not shown). In isolated liver cells, the proton leak (in the absence of nigericin) has similar characteristics and activity to that in isolated mitochondria. Thus, it appears that the use of nigericin in our study would not lead us to conclude that the proton leak in any of the isolated mitochondrial types is significant, when this is not the case in vivo. It is also probable that it does not contribute to the differences in proton leak activity in mitochondria from different tissues.

The drop in RCR of brain and muscle mitochondria in the presence of nigericin is partly due to a depression of the state 3 rate (data not shown). Thus it is possible that nigericin affects the phosphorylating system kinetics (Fig. 2b). However, it is clear from the figure that omission of nigericin would only accentuate any differences seen and would thus not affect the conclusion that isolated brain and muscle mitochondria have more active phosphorylating systems than those from liver.

The RCR of liver mitochondria is not affected by nigericin, although both the state 3 and state 4 rates are increased [46]. This equal activation implies that nigericin acts via a stimulation of the respiratory chain in liver. The respiratory chain kinetics shown in Fig. 2a indicate that mitochondria from the other organs have more active chains than liver. It is unlikely that nigericin contributes to these differences as its effect in brain and muscle mitochondria is to depress the state 3 rate by around 25–30% (data not shown). We can either conclude that nigericin does not stimulate the chain in brain and muscle mitochondria – and thus omission of nigericin from the incubation medium would accentuate the differences shown in Fig. 2a – or that it does and that its effect is counterbalanced by some inhibitory effect on the phosphorylating system (e.g., a change in matrix pH) induced by nigericin.

3.5. Control of oxidative phosphorylation

Control of oxidative phosphorylation in liver, kidney, brain and skeletal muscle was compared. This was done in both state 3 and state 4, using the 'top-down' method of metabolic control analysis as described by Brown et al. [30] and Hafner et al. [21]. Control analysis quantifies the importance of particular steps in a pathway in the control of its rate (flux control coefficients) or in the control of intermediate metabolite concentrations in the pathway (concentration control coefficients). It has been shown, using the top-down approach, that the distribution of control over respiration and oxidative phosphorylation in isolated liver

mitochondria [21] is similar to that in mitochondria within intact resting liver cells [47]. Hence control analysis in isolated mitochondria appears to be a good model for control in intact, resting tissues.

In order to analyse its control, the system of oxidative phosphorylation is conceptually divided into three blocks or branches, each connected via the same pathway intermediate ($\Delta\Psi$) as outlined by Hafner et al. [21]. The $\Delta\Psi$-producing branch (the 'substrate oxidation system') includes the dicarboxylate carrier, succinate dehydrogenase and the electron transport chain. The $\Delta\Psi$-consuming branches are (a) the proton leak – includes ion cycling by proton symport/antiport as well as any intrinsic leakiness of the membrane, and (b) the phosphorylating system – includes the adenine nucleotide carrier, phosphate carrier, ATP synthase and hexokinase. $\Delta\Psi$ was chosen as the pathway intermediate rather than Δp, as it is experimentally simpler to measure the entire Δp as $\Delta\Psi$, by converting the ΔpH component to electrical potential using nigericin. It has been shown [46] that presence or absence of ΔpH has little effect on the distribution of control over oxidative phosphorylation in liver mitochondria respiring on succinate.

The experimental protocol was as outlined by Hafner et al. [21] with appropriate controls to ensure that this was suitable for mitochondria from the other tissues (see Experimental section). Basically, the method involves changing the activity of one branch of the system, and measuring the response of the other branches to the new steady state value of $\Delta\Psi$ thus produced. The approach assumes that the branches only communicate with each other via $\Delta\Psi$. The kinetic effect of adenine nucleotides on the substrate oxidation system is the other most likely interaction. This has been shown to be negligible in isolated liver mitochondria [21]. The same is assumed, but not proved, to be true for mitochondria from other organs.

Table 3 shows the results of top-down control analysis in mitochondria isolated from liver, kidney, brain

Table 3
Control of oxidative phosphorylation in mitochondria isolated from liver, kidney, brain and skeletal muscle

	Skeletal muscle	Kidney	Brain	Liver
STATE 4:				
Control over respiration rate:				
(a) by substrate oxidation	0.39 ± 0.07	0.57 ± 0.35	0.47 ± 0.12	0.42 ± 0.06
(b) by the proton leak	0.61 ± 0.07	0.43 ± 0.35	0.53 ± 0.12	0.58 ± 0.06
Control over proton leak:				
(a) by substrate oxidation	0.39 ± 0.07	0.57 ± 0.35	0.47 ± 0.12	0.42 ± 0.06
(b) by the proton leak	0.61 ± 0.07	0.43 ± 0.35	0.53 ± 0.12	0.58 ± 0.06
Control over $\Delta\Psi$:				
(a) by substrate oxidation	0.05 ± 0.03	0.03 ± 0.01	0.08 ± 0.03	0.01 ± 0.01
(b) by the proton leak	−0.05 ± 0.03	−0.03 ± 0.01	−0.08 ± 0.03	−0.01 ± 0.01
STATE 3:				
Control over respiration rate:				
(a) by substrate oxidation	0.62 ± 0.50	0.57 ± 0.17	0.69 ± 0.02	0.44 ± 0.01
(b) by phosphorylating system	0.32 ± 0.40	0.39 ± 0.15	0.29 ± 0.01	0.53 ± 0.01
(c) by the proton leak	0.06 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	0.03 ± 0.00
Control over phosphorylation:				
(a) by substrate oxidation	0.66 ± 0.04	0.52 ± 0.13	0.70 ± 0.04	0.43 ± 0.01
(b) by phosphorylating system	0.44 ± 0.03	0.53 ± 0.12	0.36 ± 0.03	0.59 ± 0.20
(c) by the proton leak	−0.10 ± 0.01	−0.05 ± 0.01	−0.06 ± 0.01	−0.02 ± 0.00
Control over proton leak:				
(a) by substrate oxidation	0.44 ± 0.54	0.87 ± 0.36	0.58 ± 0.28	0.54 ± 0.40
(b) by phosphorylating system	−0.38 ± 0.54	−0.79 ± 0.32	−0.53 ± 0.26	−0.52 ± 0.39
(c) by the proton leak	0.94 ± 0.10	0.92 ± 0.03	0.95 ± 0.02	0.98 ± 0.01
Control over $\Delta\Psi$:				
(a) by substrate oxidation	0.09 ± 0.03	0.09 ± 0.03	0.10 ± 0.01	0.06 ± 0.02
(b) by phosphorylating system	−0.08 ± 0.02	−0.08 ± 0.03	−0.09 ± 0.01	−0.06 ± 0.02
(c) by the proton leak	−0.01 ± 0.01	−0.01 ± 0.00	−0.01 ± 0.00	−0.00 ± 0.00
Control over P/O ratio:				
(a) by substrate oxidation	0.04 ± 0.54	−0.04 ± 0.30	0.01 ± 0.06	0.01 ± 0.02
(b) by phosphorylating system	0.12 ± 0.43	0.14 ± 0.27	0.07 ± 0.04	0.06 ± 0.21
(c) by the proton leak	−0.16 ± 0.02	−0.09 ± 0.03	−0.08 ± 0.02	−0.05 ± 0.00

Data represent the mean ± S.E. ($n = 3$ independent experiments) of the control coefficients calculated from the kinetic data shown in Figs. 1 and 2, using the equations given in Hafner et al. [21] and Brand et al. [46].

and skeletal muscle. The control coefficients were calculated using the data shown in Figs. 1a, 2a and 2b as outlined by Hafner et al. [21]. The degree of control exerted by any branch of our simplified system of oxidative phosphorylation is expressed as a coefficient. Control over pathway flux is expressed as a flux control coefficient. This is the fractional change in the flux through a given branch resulting from an infinitesimally small change in the activity of the same or another branch. This gives information about the relative importance, under a certain set of conditions, of each branch of the system in controlling pathway flux. Control over the pathway intermediate $\Delta\Psi$ is expressed as a concentration control coefficient. This is the fractional change in the 'concentration' (i.e., magnitude) of $\Delta\Psi$ due to an infinitesimally small change in the activity of any one of the branches. This gives information on the homeostatic control of membrane potential. Control over the P/O ratio by any branch of the system is calculated as the difference in the control exerted by that branch over the respiration rate and the phosphorylating rate [46]. The P/O ratio in this case is the effective P/O ratio resulting from variations in the proportion of total respiration used to drive the proton leak flux, rather than the mechanistic P/O ratio calculated from H^+/O and H^+/ATP ratios.

The control over oxidative phosphorylation is analysed in non-phosphorylating (state 4) and phosphorylating (state 3) respiration. In state 4, the coefficient of control over the two pathway fluxes (respiration rate and proton leak) by the two branches of the system (the proton leak and the substrate oxidation system) has a value between zero and one, one being all the control. In state 3, the coefficient of control over two of the three pathway fluxes (phosphorylation and proton leak) by two of the three branches of the system (the proton leak and phosphorylating system) may also have a negative value. This is because the two branches compete with each other for the intermediate ($\Delta\Psi$), and increasing flux through one branch will result in a decrease in flux through the other. In both state 3 and state 4, the sum of the control coefficients of the three branches over a given pathway flux is unity, according to the summation theorem outlined by Kacser and Burns [48]. Similarly, the theoretical sum of the control coefficients of the three branches over $\Delta\Psi$ and the P/O ratio is zero since all measurements are made in the steady state. Note that the assumption that the sum of the control coefficients is one (for fluxes) and zero (for $\Delta\Psi$ and P/O) is reflected in the calculations used to produce the data in Table 3 and hence the fact that these data conform to the summation theorem is a direct consequence of the equations used for the calculation and not an experimental result.

The data in Table 3 essentially show that, for the simplified model of oxidative phosphorylation used in this analysis, the pattern of control in the different tissues is similar. The control distribution in the liver was similar to that reported by Hafner et al. [21] and Brand et al. [31] using similar conditions. Control by the phosphorylating system (0.32) and the substrate oxidation system (0.62) over state 3 respiration rate in skeletal muscle is similar to that reported by Letellier et al. (0.24–0.38 for the phosphorylating system and 0.50–0.59 for the sum of complexes I, II and III; [49]) and Wisniewski et al. (about 0.3 for sum of the ATP synthase, adenine nucleotide translocator, phosphate transporter and the ATPase used to generate steady state phosphorylation [50]) using a different approach.

Concentrating on control by the proton leak, we can see that:

(a) Control of respiration rate in kidney, brain and muscle is very similar to that in liver in both state 4 and state 3. Control by the leak over respiration rate is significant (0.21–0.22; [8,50]) in resting liver cells and respiration by the liver, kidney, brain and skeletal muscle accounts for about 75% of the total resting tissue oxygen-consumption rate in rats [3]. Hence if we made the arbitrary assumption that control over resting respiration in the other organs was similar to that in liver cells, then manipulation of the proton leak in the whole animal might lead to significant changes in resting heat production.

(b) There are three significant ways of increasing the leak flux: (i) by increasing the leak, (ii) by decreasing the activity of the phosphorylating system and (iii) by increasing the activity of the respiratory chain. Thus, for example, the role of the enhanced activity of the substrate oxidation system in isolated kidney mitochondria (Fig. 2a) might be to increase proton leak, assuming the same phenomenon is present in vivo.

(c) The proton leak is negligible in state 3. This is a manifestation of the non-linear dependence of the leak on $\Delta\Psi$, and has consequences for the stoichiometric efficiency (as measured by the number of ATP produced per mole of oxygen consumed) of oxidative phosphorylation when it is most necessary to conserve energy as ATP. The low value for the control coefficient over the effective P/O ratio by the proton leak also indicates the tight control over the stoichiometric efficiency of phosphorylation in state 3.

In conclusion, we have shown that the proton leak in mitochondria isolated from kidney, brain and skeletal muscle has similar characteristics to that of liver mitochondria. We have also observed that the distribution of control over oxidative phosphorylation in state 3 and state 4 is similar in all the tissues analysed. This work supports the hypothesis [11] that proton leak is significant in different intact tissues and therefore that proton leak may be an important contributor to resting

metabolic rate. Further work using intact tissues is required before this contribution can be quantified.

Acknowledgements

This work was supported by a research studentship (to D.F.S.R.) and a grant (to M.D.B.) from the Agriculture and Food Research Council. Thanks to Mark Leach for technical assistance and Richard Porter and Mary-Ellen Harper for helpful discussions.

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