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Evolution of energy metabolism

Proton permeability of the inner membrane of liver mitochondria is greater in a mammal than in a reptile

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Standard metabolic rate is 7-fold greater in the rat (a typical mammal) than in the bearded dragon, *Amphibolurus vitticeps* (a reptile with the same body mass and temperature). Rat hepatocytes respire 4-fold faster than do hepatocytes from the lizard. The inner membrane of isolated rat liver mitochondria has a proton permeability that is 4–5-fold greater than the proton permeability of the lizard liver mitochondrial membrane per mg of mitochondrial protein. The greater permeability of rat mitochondria is not caused by differences in the surface area of the mitochondrial inner membrane, but differences in the fatty acid composition of the mitochondrial phospholipids may be involved in the permeability differences. Greater proton permeability of the mitochondrial inner membrane may contribute to the greater standard metabolic rate of mammals.

INTRODUCTION

The rate at which a resting animal carries out metabolism is determined mainly by its phylogenetic group, thyroid-hormone level, temperature and body size; the standard metabolic rate (M) of vertebrates depends on size according to the equation $M = aW^{0.75}$, where W is body mass. The value of the elevation constant, a, is about 4–5-fold higher in homoeothermic animals (mammals and birds) than it is in reptiles and other poikilotherms, so that a resting mammal consumes oxygen 4–5 times as fast as a reptile of the same body mass, if the comparison is made at the same body temperature (Hemmingsen, 1960; Dawson & Hulbert, 1970). To investigate the biochemical mechanisms involved in this difference between mammals and reptiles, we need to minimize differences in body size and temperature and compare otherwise similar species. The bearded dragon (*Amphibolurus vitticeps*), an agamid lizard found in central Australia, is a suitable reptile for comparison with the laboratory rat (Else & Hulbert, 1987; Hulbert & Else, 1989), since its preferred body temperature is around 37 °C (Bartholemew & Tucker, 1963) and it has a similar body mass. In the same way, the central netted dragon (*A. nuchalis*) has been compared with the mouse (Else & Hulbert, 1981; Hulbert & Else, 1981).

The difference in standard metabolic rate between mammals and reptiles is retained at the level of the isolated organ, since liver slices from mice or rats consume oxygen about 5 times faster than liver slices from the corresponding lizards (Hulbert & Else, 1981; Else & Hulbert, 1987). It is also reflected in the amount of mitochondrial inner membrane in the animals; mammals have slightly more inner membrane per cm^3 of mitochondrial matrix, more volume of matrix per cell in several different organs, and more mass of these organs per unit body mass, giving a severalfold greater surface area of mitochondrial inner membrane in the metabolically active internal organs than for the corresponding reptiles (Else & Hulbert, 1981, 1985*a*). They also have a greater cell, organ and total content of mitochondrial enzymes and cytochromes (Bennett, 1972; Hulbert & Else, 1989). Thus the differences in standard metabolic rate of whole animals are paralleled by a difference in the total amount of mitochondrial inner membrane.

Mitochondria make ATP by pumping protons across the inner membrane during electron transport; the protonmotive force so generated drives protons back to the matrix through the proton-translocating ATP synthase, and ATP is synthesized. Other non-productive pathways for the return of the protons to the matrix (proton leaks) exist in isolated mitochondria (Nicholls, 1974; Krishnamoorthy & Hinkle, 1984; Brown & Brand, 1986), and cause heat production and lowered efficiency of oxidative phosphorylation (Brand & Murphy, 1987; Murphy, 1989; Brand, 1990*b*). The leak of protons across the mitochondrial inner membrane has been shown to occur in intact rat hepatocytes, where it accounts for about 20–25% of the oxygen consumption under resting conditions (Nobes *et al.*, 1990; Brown *et al.*, 1990), leading to the hypothesis that this process may be a significant contributor to standard metabolic rate (Brand, 1990*a*).

The thyroid gland of mammals is considerably more active than the reptilian thyroid (Hulbert & Williams, 1988), and many of the previously reported metabolic differences (such as their standard metabolic rates, amounts of mitochondrial membrane, tissue ion permeabilities and pumping etc.) between reptiles and mammals are similar to the differences caused by thyroid hormones in mammals (Hulbert, 1987). In some respects, mammals could be regarded as very hyperthyroid reptiles, although this cannot completely explain the metabolic differences between these groups. The leak of protons across the mitochondrial inner membrane is increased in hepatocytes from euthyroid rats compared with hypothyroid ones (Nobes *et al.*, 1990) and there is a dramatic effect of thyroid hormone on the proton leak in isolated mitochondria, with a 7-fold increase in the leak in mitochondria isolated from hyperthyroid rats compared with mitochondria isolated from hypothyroid rats (Hafner *et al.*, 1988, 1989).

This background, together with a report by Akhmerov (1986) that mitochondria from frog liver and heart were less leaky to protons than mitochondria from the rat, led us to make a comparison in rats and bearded dragons of the respiration rate

Abbreviation used: TPMP, methyltriphenylphosphonium.

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Table 1. Oxygen uptake by whole animals and by hepatocytes from rats and lizards

Lizard hepatocytes were incubated in medium containing 137 mM-NaCl, 5.4 mM-KCl, 0.8 mM-$MgCl_2$, 0.85 mM-Na_2HPO_4, 0.15 mM-KH_2PO_4, 1 mM-$CaCl_2$ and 15 mM-glucose. Non-mitochondrial respiration was measured in the presence of 2 μg of antimycin/ml. An upper limit to respiration driving the proton leak was estimated as respiration rate in the presence of 20 μg of oligomycin/ml minus the respiration rate in the presence of antimycin. A lower limit to respiration driving ATP synthesis was estimated as control respiration rate minus respiration rate in the presence of oligomycin [see Nobes *et al.* (1990) and Brand (1990*b*)]. Respiration driving the Na^+/K^+-ATPase was measured as control respiration rate minus respiration rate 6 min after the addition of 1.5 mM-ouabain (see Nobes *et al.*, 1989). All values are expressed as means ± S.E.M. (or S.D. where indicated) for the numbers of animals in parentheses. Data for rat hepatocyte respiration rate are taken from Nobes *et al.* (1990), assuming that 60% of dry weight of hepatocytes is protein; data for the processes contributing to rat hepatocyte respiration are taken from Brand (1990*b*), Brown *et al.* (1990) and Nobes *et al.* (1989, 1990).

	R. norvegicus (rat)	*A. vitticeps* (lizard)
Standard metabolic rate (ml of O_2/h per g)	0.779 ± 0.028 (5)	0.109 ± 0.011 (6)
Body wt. (g)	304 ± 34 (11) (S.D.)	275 ± 102 (11) (S.D.)
Liver wt. (g)	10.1 ± 2.2 (4) (S.D.)	5.4 ± 1.8 (5) (S.D.)
Hepatocyte respiration rate (nmol of O/min per mg of protein)	10.7	2.6 ± 0.9 (6)
Hepatocyte respiration rate to drive the following processes (% of total hepatocyte rate):		
Non-mitochondrial	14–23	15.7 ± 4.3 (6)
Proton leak	19–25	< 30.1 ± 3.4 (3)
ATP synthesis	56–64	> 54.1 ± 7.1 (3)
[including Na^+/K^+-ATPase	6–10	10 (1)]

and proton leak in hepatocytes and isolated liver mitochondria. The results show that mitochondria from the mammal are about 4–5-fold more permeable to protons than are mitochondria from the reptile.

EXPERIMENTAL

Amphibolurus vitticeps were collected in summer in North-western New South Wales and maintained at 37 °C for at least 3 weeks before use. Rats and lizards were kept essentially as described by Else & Hulbert (1987). Rats were provided with access *ad lib.* to a complete diet, and lizards were fed on fruit, vegetables and mealworms daily. All animals had access *ad lib.* to drinking water.

The standard metabolic rates of both rats and lizards were measured by using an open-flow system, in which the flow of dry CO_2-free air was measured and controlled by a calibrated Hastings Teledyne Mass Flow Controller and the oxygen content of inlet and dry CO_2-free exit air was measured with a Taylor Servomex type OA272 oxygen analyser and continuously recorded on a National Chart recorder. Ambient temperature was maintained at 30 °C for the rats and at 37 °C for the lizards (in order that their body temperature was 37 °C). All animals were fasted overnight before oxygen-consumption measurements.

Lizard hepatocytes were prepared as described by Else & Hulbert (1987). For preparation of liver mitochondria, animals were weighed and then decapitated. The liver was removed into ice-cold isolation medium [250 mM-sucrose, 10 mM-Tris/HCl, 1 mM-(K)EGTA, pH 7.4], chopped finely, and homogenized in a glass Dounce homogenizer. The supernatant from a 3 min centrifugation at 3000 rev./min in a Sorvall centrifuge was re-centrifuged for 10 min at 10000 rev/min, and the more mobile upper layer of pellet was resuspended and re-centrifuged twice. Care was taken to discard as much as possible of the dense black lower layer obtained from the lizards, as this contained no mitochondria. The final mitochondrial pellet was resuspended to about 50 mg of protein/ml in isolation medium and kept on ice until use. Protein was assayed by the Lowry method, with bovine serum albumin as a standard.

Mitochondrial oxygen consumption and membrane potential were measured in triplicate for all points, at 1 mg of mitochondrial protein/ml in incubation medium containing 120 mM-KCl, 5 mM-Hepes, pH 7.2, 1 mM-(K)EGTA, 5 μM-rotenone, 2 μg of oligomycin/ml, 0.4 μg of nigericin/ml, 5 μM-methyltriphenylphosphonium (TPMP) and 10 mM-(K) succinate at 37 °C. Different steady states were established by addition of 1 M-(K) malonate to give final concentrations of 0.33, 0.67, 1, 2, 3, 5 or 10 mM. Respiration rates were measured in a thermostatically regulated 3 ml chamber containing a Clarke-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). Membrane potential was measured in parallel incubations using [^{3}H]TPMP as described by Brown & Brand (1985). Correction for binding of TPMP was determined by using [^{3}H]TPMP and ^{86}Rb at different matrix volumes (set by varying sucrose concentration and determined with [^{14}C]sucrose and ^3H_2O) as described by Brown & Brand (1988). The volume of glutaraldehyde-fixed mitochondrial pellets (Table 2) was measured in the same way, by using ^3H_2O, but the pellet was allowed to remain in scintillant for at least 72 h before assay, by which time radioactivity had escaped from the solid pellet and the counts were stable. Radioactivity was assayed by dual-label counting in an LKB Rackbeta liquid-scintillation counter with appropriate quench corrections.

Mitochondrial pellets for electron microscopy were prepared in isolation medium containing 2.5% (v/v) glutaraldehyde for both lizards and rats. The pellets were then treated as tissue blocks, by using the techniques of fixation, electron microscopy and stereology described previously for tissues by Else & Hulbert (1985*a*). Mitochondrial membrane surface was determined by using 13–20 electron micrographs from each mitochondrial pellet. The standard errors of the determination for each pellet averaged 1.6% and 1.4% of the individual mean values for the reptiles and mammals respectively. In previous studies we have differentiated between cristae-membrane surface area and inner-membrane surface area; in this publication inner-membrane surface area refers to the sum of these two membrane surface areas.

The fatty acid composition of mitochondrial phospholipids from each species was ascertained by methods described previously (Hulbert & Else, 1989). Basically, total lipids were extracted from mitochondrial preparations with chloroform/

methanol (2:1, v/v) and phospholipids were separated by silicic acid column chromatography. Phospholipid fatty acids were methylated by using BF_3 in methanol, and their relative composition was quantified by g.l.c.

Biochemicals were from Sigma; ^3H_2O, [^{14}C]sucrose and ^{86}Rb were from Amersham Radiochemicals, and [^{3}H]TPMP was from New England Nuclear (Du Pont).

RESULTS

The standard metabolic rate of *A. vitticeps* was 0.109 ml of O_2/h per g at a body temperature of 37 °C, which is 14% of the level of standard metabolism measured for the rat (with an assumed body temperature of approx. 37 °C). These measured values (Table 1) are similar to those previously reported for the rat (Kleiber, 1961) and for the closely related lizard species *A. barbatus* (Bartholemew & Tucker, 1963). Thus these species can be regarded as reasonably typical of their respective groups in that the metabolic activity of the mammal is several times (7 times in the present comparison) that of a reptile of the same size and at the same body temperature.

Else & Hulbert (1987) found that the difference in respiration rates was still apparent in liver slices from these animals; rat liver slices had a respiration rate that was more than 4-fold higher than the respiration rate of lizard liver slices. Table 1 shows that this difference persists in isolated hepatocytes; rat hepatocytes (Nobes *et al.*, 1990) respire at about 4 times the rate of lizard hepatocytes. The respiration rate of the lizard hepatocytes converts to 1.04 μl of O_2/h per mg dry wt. (assuming 60% of dry wt. is contributed by protein), agreeing with the value for lizard liver slices (Else & Hulbert, 1987).

Nobes *et al.* (1989, 1990) measured respiration and mitochondrial potential in rat hepatocytes treated with different inhibitors, and quantified the contribution of several processes to oxygen consumption. We carried out a less extensive analysis to show that the relative importance of different processes in lizard hepatocytes is generally similar. Because of the low yield of hepatocytes from the lizards, mitochondrial membrane potential was not measured, so the value in Table 1 for the proportion of oxygen consumption devoted to driving the mitochondrial proton leak in intact lizard hepatocytes is a maximum estimate, and the value for the proportion devoted to ATP synthesis is a minimum estimate. This is because addition of oligomycin to inhibit the mitochondrial ATP-synthesis reaction will increase the mitochondrial potential and so increase the potential-dependent proton leak, giving an overestimate of the oxygen consumption that was being used to drive the cycle of proton pumping and leak across the mitochondrial inner membrane and an underestimate of the oxygen consumption that was being used to drive ATP synthesis (see Brand, 1990*b*).

Table 2 reports the properties of liver mitochondria isolated from the two species. The respiratory control ratios were similar, but the mitochondria from the lizard had lower resting and maximal respiration rates and a higher resting membrane potential. The lipophilic cation TPMP was used to measure membrane potential; control experiments showed that binding of the probe was the same in liver mitochondria from the two species (Table 2). All membrane potentials reported here were calculated by using these corrections for TPMP binding.

The kinetics of the leak of protons across the mitochondrial inner membrane can be easily measured by titration of respiration rate with inhibitors of electron transport and simultaneous measurement of membrane potential, as discussed by Brand (1990*b*). The rate of proton leak is a linear function of the respiration rate. This assertion requires that the number of protons pumped by the electron-transport chain at different potentials is the same; there is increasing evidence that this is the case (Brown, 1989; Hafner & Brand, 1991). The proton leak is driven by the membrane potential, so a plot of respiration rate as a function of membrane potential is also a plot of the rate of proton leak as a function of the driving force, or a current–voltage curve for proton leak across the inner membrane. Fig. 1 shows that the leak of protons across the inner membrane of liver mitochondria from the lizard is considerably less than the corresponding leak across rat liver mitochondria. At comparable potentials the permeability to protons is about 4–5-fold greater in the rat than in the lizard.

The observation than rat liver mitochondria are leakier to protons than lizard liver mitochondria is reminiscent of the differences in mitochondrial proton leak in rats caused by thyroid hormones (Hafner *et al.*, 1988, 1989). The underlying reason for this increase in proton conductance per mg of mitochondrial protein in response to thyroid hormones is not known, but among possible explanations it could be caused by an increase in the mitochondrial inner-membrane surface area per mg of protein or by changes in the permeability of the membrane per unit area

Table 2. Properties of liver mitochondria from rats and lizards

For details see the Experimental section. Oxygen consumption was measured with an oxygen electrode at 37 °C in medium containing 1 mg of mitochondrial protein/ml, 120 mM-KCl, 5 mM-Hepes, pH 7.2, 1 mM-(K)EGTA, 5 μM-rotenone, 2 μg of oligomycin/ml, 0.4 μg of nigericin/ml, 5 μM-TPMP and 10 mM-(K) succinate. Respiratory control ratio was calculated as respiration rate in the presence of 1 μM-carbonyl cyanide *m*-chlorophenylhydrazone divided by the non-phosphorylating rate. For electron microscopy, mitochondria (1 mg of protein/ml) were suspended in medium containing 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.4, 1 mM-(K)EGTA and 2.5% glutaraldehyde at 25 °C for 15 min, then spun in a bench centrifuge to give a fixed pellet. All values are expressed as means ± S.E.M. for the numbers of animals in parentheses.

	R. norvegicus (rat)	*A. vitticeps* (lizard)
Respiratory control ratio	4.4 ± 0.6 (5)	3.4 ± 0.3 (7)
Non-phosphorylating respiration rate (nmol of O/min per mg of protein)	43.7 ± 1.7 (3)	35.1 ± 3.2 (4)
Non-phosphorylating membrane potential (mV)	179.2 ± 2.3 (3)	195.3 ± 0.9 (4)
TPMP binding correction	0.64 (3)	0.69 (3)
Volume of fixed pellet (μl/mg of protein)	4.68 ± 0.06 (6)	7.29 ± 0.2 (5)
Surface area of inner membrane:		
(m^2/cm^3 fixed pellet)	13.9 ± 0.4 (4)	8.3 ± 0.4 (3)
(m^2/g of protein)	65.1 ± 2.0 (4)	60.2 ± 3.6 (3)

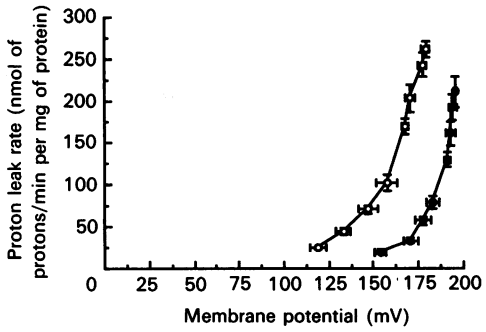

Fig. 1. Kinetics of proton leak in liver mitochondria from rats (○) and lizards (●)

Mitochondria were suspended at 37 °C in medium containing rotenone (to prevent oxidation of other substrates), succinate (respiratory substrate), oligomycin (to prevent ATP synthesis) and nigericin (to clamp the transmembrane pH gradient at zero and allow the protonmotive force to be expressed entirely as membrane potential), and membrane potential and respiration rate were titrated with malonate as described in the Experimental section. Proton leak rate was calculated from respiration rate by assuming six protons pumped (and leaked) per oxygen atom. Data represent means ± S.E.M. for 3 (rat) or 4 (lizard) independent experiments.

Table 3. Fatty acid composition of phospholipids of isolated liver mitochondria from rats and lizards

Values are mole percentages of total fatty acids expressed as means ± S.E.M. (for $n = 3$ rats or $n = 6$ lizards). Fatty acids are designated by the number of carbon atoms:number of double bonds (position of most distal double bond). Unsaturation index is the average number of double bonds per 100 fatty acids.

Fatty acid	*R. norvegicus* (rat)	*A. vitticeps* (lizard)
$C_{14:0}$	1.4±0.9	0.3±0.2
$C_{16:0}$	13.4±2.6	18.4±3.7
$C_{18:0}$	26.0±0.9	17.2±1.7
$C_{14:1}$	5.9±0.2	4.3±1.8
$C_{16:1}$	2.0±0.9	2.2±0.8
$C_{18:1(n9)}$	4.1±0.8	17.6±2.6
$C_{18:1(n7)}$	2.6±0.6	1.4±0.7
$C_{18:2(n6)}$	6.2±1.4	21.0±3.7
$C_{18:3(n3)}$		0.9±0.2
$C_{20:3(n6)}$	0.7±0.1	0.7±0.2
$C_{20:4(n6)}$	26.4±1.9	8.7±0.8
$C_{20:5(n3)}$		0.6±0.2
$C_{22:5(n6)}$	0.3±0.3	
$C_{22:5(n3)}$	0.7±0.0	
$C_{22:6(n3)}$	10.5±1.6	
% total unsaturates	59.5±3.4	56.5±4.5
% monounsaturates	14.7±1.9	24.7±3.4
% *n*6 polyunsaturates	33.7±3.3	30.4±4.0
% *n*3 polyunsaturates	11.2±1.5	1.5±0.2
Unsaturation index	203±18	109±8
$C_{20:4}/C_{18:2}$	4.7±1.0	0.5±0.1
Average chain length	15.8±0.7	17.6±0.2

following changes in the fatty acid composition of the membrane phospholipids. We therefore examined the surface area and fatty acid composition of rat and lizard mitochondria.

The area of the mitochondrial inner membrane per mg of protein was measured by pelleting mitochondria in glutaraldehyde and measuring the surface-area/volume ratio in electron micrographs and the volume/protein ratio (by using radiolabelled probes) in the pellets. The results are shown in Table 2. The surface area of mitochondrial inner membrane per mg of protein was less than 10% greater in rat mitochondria, showing that the very large differences in proton permeability cannot be accounted for by the small changes in the area of membrane. The value for the rat is comparable with that reported for isolated mitochondria by Schwerzmann *et al.* (1986), and the absolute values and the small differences between reptile and mammal are similar to those reported for intact muscle of the Cuban Iguana (*Cyclura nubila*) by Conley *et al.* (1989).

The fatty acid composition of phospholipids in whole liver (and kidney) from the rat and this lizard species differs, the basic difference being that membrane fatty acids in the rat liver contain significantly greater amounts of the long-chain polyunsaturated fatty acids (Hulbert & Else, 1989). This difference is more pronounced when we compare the phospholipid fatty acid composition of isolated liver mitochondria from these two species (Table 3). Phospholipids from isolated rat liver mitochondria have the same proportion of unsaturated fatty acids as those from lizard liver mitochondria, but the mammalian phospholipids are much more unsaturated, with an unsaturation index of 203, compared with 109 for the reptilian liver mitochondria. This difference is largely (but not completely) due to the greater amount of *n*3 polyunsaturates (ω3 fatty acids) in the rat mitochondrial membranes. Apart from the considerably greater amount of *n*3 fatty acids, within both the *n*6 and *n*3 fatty acids there is tendency for the mammalian fatty acids to be considerably more unsaturated. This is illustrated by the ratio of $C_{20:4}/C_{18:2}$, which gives an indication of desaturase activity. In the rat mitochondria there is about 4.7 times more $C_{20:4}$ (arachidonic acid) than there is $C_{18:2}$ (linoleic acid), whereas in the reptilian mitochondria the corresponding value is 0.47. Within the *n*3 fatty acids, the long-chain polyunsaturated docosahexanoic acid ($C_{22:6}$) is absent for reptilian mitochondria, but is 10.5% of total phospholipid fatty acids in rat liver mitochondria.

DISCUSSION

The results presented here show that the inner membrane of liver mitochondria from the lizard *A. vitticeps* is severalfold less permeable to protons than the inner membrane of liver mitochondria from the rat. Since both species appear to be typical of their groups in terms of mitochondrial density and metabolic rate (Else & Hulbert, 1985*a*,*b*), we propose that this difference is general for reptiles compared with mammals, and perhaps for poikilotherms compared with homoeotherms.

Previous comparisons of mitochondria from ectotherms and endotherms have not uncovered any difference in proton permeability (e.g. Cassuto, 1971), because the experiments were not designed to test for such a difference. Akhmerov (1986) reported that heart and liver mitochondria from an amphibian had lower state-4 (non-phosphorylating) respiration rates than those from rat, but as an explanation he postulated a physiologically important subpopulation of completely proton-permeable mitochondria in the mammal. Akhmerov (1986) did not report the species of frog used, nor the body weight, and compared frogs maintained at 20–22 °C with rats, so that two important variables, body temperature and body mass, were not properly controlled. His results could be interpreted as showing differential damage during preparation of rat mitochondria compared with frog mitochondria, particularly since his preparation of rat heart mitochondria had a respiratory control ratio of only 1.6 on succinate, compared with a ratio of 3.8 for frog heart mitochondria (although such a difference was not evident with the liver mitochondria). Nonetheless, it seems likely that the phenomenon observed by Akhmerov (1986) in frogs has a similar basis to the

one we report here, namely a lower proton permeability (compared with mammals) of the inner membrane of all the mitochondria in the preparation rather than a lower proportion of completely uncoupled mitochondria. The possibility that the titrations of respiration rate and membrane potential that we have carried out are non-linear not because of a dependence of proton conductance on protonmotive force, but because of the presence of a subpopulation of completely proton-permeable mitochondria, was suggested by Duszynski & Wojtczak (1985), but has been shown to be untrue (Brown & Brand, 1986; Zolkiewska *et al.*, 1989).

The respiratory control ratios that we report here are lower than those that we could have obtained with the same mitochondria had we carried out the assays under more optimal conditions *in vitro*, i.e. at 25 °C instead of 37 °C, and in the absence of nigericin. Nevertheless, our finding that the lizard mitochondria had a slightly lower respiratory control ratio than the rat mitochondria, despite being intrinsically less leaky to protons, provides indirect evidence that the greater proton permeability in rats is not an artefact of a poorer mitochondrial preparation. This conclusion also follows from previous observations that the proton leak across the mitochondrial inner membrane in intact rat hepatocytes is similar in magnitude to the proton leak in isolated rat liver mitochondria (Nobes *et al.*, 1990).

Although the absolute rate of oxygen consumption needed to drive the mitochondrial proton leak in lizard hepatocytes was about one-third of the rate needed in rat hepatocytes, we did not observe that the proton leak required a smaller proportion of the total respiration rate in lizard cells (Table 1). This might be because the protonmotive force is maintained at a higher value in lizard hepatocytes, so that, despite the decreased conductance to protons, the proton leak rate is not corresponding decreased. Alternatively, it might be because our measurement of mitochondrial proton leak in lizard hepatocytes is a maximum estimate (because we did not correct for the increase in protonmotive force that would be expected to follow inhibition of ATP synthesis, owing to the difficulty of measuring mitochondrial potential in these cells because of poor yield of cells), and the true proportion of oxygen consumption used to drive this process might be smaller in lizards than in rats. If the non-phosphorylating membrane potentials of isolated mitochondria (Table 2) are indicative of the situation *in vivo*, then the first alternative is likely to be at least part of the explanation.

We have considered two potential mechanisms which might be responsible for the greater proton permeability of rat mitochondria: increased surface area per mg of protein, and altered fatty acid composition of membrane phospholipids. It is clear that the first potential mechanism cannot be the explanation for the difference in proton permeability between rat and lizard mitochondria, since the differences in surface area are far too small to account for the large differences in proton permeability (Table 2 and Fig. 1).

The second potential mechanism involves the nature of the fatty acid side chains of the phospholipids of the mitochondrial membrane. Manipulation of yeast mitochondrial membrane fatty acids has been shown to result in changes in the proton permeability and phosphorylating ability (Haslam *et al.*, 1971). We have shown here (Table 3) that there are substantial differences in the membrane fatty acids between lizard and rat mitochondria and suggest that these may be related to the increased proton permeability of the mammalian mitochondria. The difference in the content of *n*3 fatty acids between the lizard and rat mitochondria may be quite important. We calculate that in the middle of the mitochondrial membrane (specifically, approx. 30% of the fatty acid portion of the bilayer) there are 7–8 times the number of double bonds in the rat mitochondria than in the lizard mitochondria (11.2 double bonds per 100 fatty acids in the rat compared with 1.5 double bonds per 100 fatty acids in the lizard mitochondria). These are likely to result in big differences in the packing of the fatty acids both within themselves and with membrane proteins, and this may be an explanation for the greater proton leak in the mammalian mitochondria, either by increasing the passive proton permeability of the bilayer itself or by influencing the behaviour of mitochondrial membrane proteins (or both). Verification of this hypothesis, however, awaits more detailed experimentation.

The increased proton permeability of mitochondria in the rat compared with the lizard has an analogy at the plasma membrane. It has been demonstrated that rat liver cells are considerably more leaky to K^+ and Na^+ than are liver cells from *A. vitticeps* (Else & Hulbert, 1987), and it has been postulated that this is also connected to a difference in plasma-membrane fatty acid composition (Hulbert & Else, 1989, 1990).

Thyroid hormones increase the mitochondrial proton leak in rats (Hafner *et al.*, 1988, 1989; Horrum *et al.*, 1990), increase the surface-area/volume ratio of the mitochondria (Gustafsson *et al.*, 1965; Jacovcic *et al.*, 1978) and also change the fatty acid composition of mitochondrial membranes (Hulbert *et al.*, 1976; Hulbert, 1978; Withers & Hulbert, 1987; Hoch, 1988). The activity of the thyroid is greater in mammals than it is in reptiles (Hulbert & Williams, 1988), and this is a probable partial explanation of the difference observed in the present study.

Our results show that liver mitochondria isolated from rats are 5-fold more permeable to protons than those isolated from lizards; this difference is predominantly due to a greater proton flux per unit area of membrane rather than to a much larger area of mitochondrial membrane per mg of protein. Whether this increased permeability leads to an increased proton leak flux in mitochondria *in situ* in liver cells is not known, since, for example, the flux through the leak pathway depends on the value of the mitochondrial membrane potential, and this is not known for lizard hepatocytes. However, from prior information on the two species, we can make some assessment of the situation. Isolated rat hepatocytes have a resting oxygen consumption (per mg of protein) that is 410% that of lizard hepatocytes (Table 1). Since liver from the rat has a protein content that is 180% of that for lizard liver per g wet weight (Hulbert & Else, 1989), the resting oxygen consumption of rat hepatocytes may be relatively even greater on a wet-weight basis. Similarly, rat liver slices have an oxygen-consumption rate (per mg dry weight) that is 430% of that measured in lizard liver slices (Else & Hulbert, 1987). Yet 1 g of liver tissue in mammals has only an average of 213% of the mitochondrial membrane of reptilian liver tissue (Else & Hulbert, 1985*a*). Since the proportion of total oxygen consumption that is mitochondrial is similar in rat and lizard hepatocytes (Table 1), it then appears that rat liver mitochondria have a resting oxygen consumption *in situ* per unit membrane surface that is twice that of lizard liver mitochondria. A much greater proton permeability of the rat liver mitochondrial membrane *in situ* must be a prime candidate to explain this difference. A qualitatively similar conclusion can be drawn from comparison of the oligomycin-insensitive respiration rates of rat and lizard hepatocytes reported in Table 1.

The proton leak is a significant contributor to the resting metabolic rate of isolated rat hepatocytes (Nobes *et al.*, 1990). The liver is a significant contributor to basal metabolism; it is estimated to be responsible for 20% of the standard metabolic rate of the rat (Jansky, 1965). Many of the metabolic differences between the rat and the lizard observed in the liver are similarly observed in other tissues (Else & Hulbert, 1985*a*, 1987; Hulbert & Else, 1989) and these two species are typical of mammals and

reptiles respectively, so it is tempting to speculate that mitochondria in most or all mammalian tissues are leakier to protons than are those of the equivalent reptilian tissues and that such an increased mitochondrial proton leak is one of the important mechanisms (though not the only one) underlying the dramatic increase in basal metabolism during the evolution of endothermy in mammals (Brand, 1990*a*).

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