Changes in permeability to protons and other cations at high proton motive force in rat liver mitochondria

Guy C. BROWN and Martin D. BRAND

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

1. We have confirmed that the respiration rate of rat liver mitochondria can be substantially inhibited with only a small drop in proton motive force. We have directly measured the passive proton permeability as a function of $\Delta\psi$ by using K^+ diffusion potentials and have shown that there is a large increase in proton permeability at high $\Delta\psi$. This can quantitatively account for the inhibitor titrations of respiration.
2. $\Delta\psi$ and ΔpH were shown to have roughly equal effects on the relatively high respiration rate in static head.
3. The permeabilities to K^+ , tetramethylammonium⁺ and choline⁺ were shown to increase greatly at high $\Delta\psi$, in a similar way to proton permeability, indicating a similar mechanism of entry.

INTRODUCTION

Nicholls (1974) was the first to demonstrate that when isolated rat liver mitochondria respiring on succinate are titrated with a respiratory inhibitor, respiration can be substantially inhibited with only a small drop in the proton motive force (Δp). Similar non-proportional relationships between electron transport rate and Δp (or $\Delta\psi$) have been observed in brown adipose tissue mitochondria (Nicholls, 1977), Jerusalem artichoke mitochondria (Mandolino *et al.*, 1983), bovine heart submitochondrial particles (Sorgato & Ferguson, 1979), vesicles of *Paracoccus denitrificans* (Kell *et al.*, 1978), and intact cells and chromatophores of *Rhodospseudomonas capsulata* (Clark *et al.*, 1983; Cotton *et al.*, 1984).

Two main explanations have been suggested for this behaviour: (a) a non-ohmic increase in proton permeability at high Δp (Nicholls, 1974), and (b) a H^+/O stoichiometry drop ('redox slippage') at high Δp (Pietrobon *et al.*, 1981).

That the proton permeability of rat liver mitochondria does increase disproportionately with $\Delta\psi$ has been shown by directly measuring the proton influx rate induced by K^+ diffusion potentials in valinomycin-treated mitochondria (Krishnamoorthy & Hinkle, 1984; O'Shea *et al.*, 1984). Moreover, the non-ohmic conductance is found in vesicles of mitochondrial lipids in the absence of protein (Krishnamoorthy & Hinkle, 1984), and is a general property of phospholipid bilayers (Gutknecht, 1984). However it has not been shown for liver mitochondria that the proton permeability characteristics can quantitatively account for the inhibitor titrations of respiration rate and Δp . Nor is it known which component of Δp ($\Delta\psi$ or ΔpH) is more important in controlling the steady state respiration rate.

Cotton *et al.* (1984) have shown that inhibitor titrations of electron transport rate and $\Delta\psi$ in chromatophores of *Rhodospseudomonas capsulata* are similarly non-linear, and that this is due to an increase in both proton and other ion permeabilities at high $\Delta\psi$. The

permeability of mitochondria to a number of ions is known to change in different states of energization, and Brierley (1978) has suggested that the electrophoretic cation permeability may be 'voltage gated'. Thus it might be that the mitochondrial permeability to ions other than protons is also non-ohmic, and might share a common mechanism.

In the present paper we show that the proton permeability characteristics of rat liver mitochondria can quantitatively account for the inhibitor titrations of respiration rate and Δp , and that both $\Delta\psi$ and ΔpH control the relatively high respiration rate in static head (the steady state of minimum electron flux and maximum Δp). We also show that the permeability of mitochondria to a number of cations increases at high $\Delta\psi$, in a similar way to proton permeability, implying a similar mechanism of entry.

EXPERIMENTAL

Preparation of mitochondria

Rat liver mitochondria were prepared according to standard methods (Chappell & Hansford, 1972), from 2–3-month-old female Wistar rats, 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 with the biuret method (Gornall *et al.*, 1949), with bovine serum albumin as a standard.

Measurement of matrix volume, ΔpH , and $\Delta\psi$

These were measured as described in Brown & Brand (1985). Mitochondrial matrix volume was calculated as $^3H_2O - [^{14}C]$ sucrose space. ΔpH was calculated from the accumulation ratio (ratio of concentration inside to concentration outside) of $[^3H]$ acetate or $[^{14}C]$ methylamine. $\Delta\psi$ was calculated from the accumulation ratio of TPMP⁺, monitored either with $[^3H]$ TPMP or with a TPMP⁺-sensitive electrode. All calculations, isotope

Abbreviations used: $\Delta\psi$, electrical potential across mitochondrial inner membrane; ΔpH , pH difference across mitochondrial inner membrane; Δp , proton motive force across mitochondrial inner membrane; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMA, tetramethylammonium; TPMP, methyltriphenylphosphonium.

activities, and methods are as in Brown & Brand (1985). Note that we calculate $\Delta\psi$ as:

$$\Delta\psi = 59 \cdot \log(0.33 \cdot \text{accumulation ratio for TPMP}^+)$$

where 0.33 is a binding correction, derived from a comparison of TPMP⁺ and Rb⁺ accumulations, which we find to be linearly related up to at least 160 mV, and have assumed to be so related at higher potentials. All measurements of $\Delta\psi$, ΔpH and matrix volume were done in parallel, each in at least triplicate for each point determination.

Measurement of respiration rates

Oxygen concentration was measured polarographically with a Clark electrode with a Teflon membrane, enclosed in a thermostatically controlled vessel.

Cation permeabilities

Cation permeabilities as a function of $\Delta\psi$ were estimated from the initial rate of swelling of mitochondria on addition of succinate, in the acetate salt of the cation. Initial swelling rates were assayed using the initial rate of absorbance decrease at 540 nm on addition of succinate, in a single beam spectrophotometer, always starting from the same initial absorbance.

Materials

[¹⁴C]Sucrose, [¹⁴C]methylamine [³H]acetate, and ³H₂O were from Amersham International (Amersham, Bucks., U.K.). [³H]TPMP bromide was from New England Nuclear Chemicals (Dreieich, Germany). Myxothiazol was from Boehringer Mannheim (Mannheim, Germany). TPMP bromide was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). All other chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.).

RESULTS

Proton permeability as a function of $\Delta\psi$

To measure the mitochondrial proton permeability directly, as a function of $\Delta\psi$, we measured the initial rate of proton influx into mitochondria, following addition of valinomycin, in the presence of different external K⁺ concentrations, as outlined by Krishnamoorthy & Hinkle (1984). The addition of valinomycin induces a diffusion potential as K⁺ effluxes from the mitochondria, and this $\Delta\psi$ was followed simultaneously with a TPMP⁺-sensitive electrode present in the medium. The initial TPMP⁺ uptake slowly decayed as the internal K⁺ effluxed into the medium and the mitochondria shrank; thus the TPMP⁺ uptake was extrapolated back to the time of valinomycin addition, as shown in Fig. 1. The initial rate of proton influx was followed with a pH electrode, calibrated *in situ* with small pulses of 0.1M-HCl. The external K⁺ concentration was varied with 0–1 mM added KCl. Rotenone, myxothiazol, oligomycin, *N*-ethylmaleimide and EGTA were present in the medium to reduce active proton translocation and any other compensating ion movements, other than that due to the passive proton permeability.

The initial rates of proton influx are plotted against the measured induced $\Delta\psi$ in Fig. 2. The graph indicates a non-ohmic rise in passive proton flux at high $\Delta\psi$, in agreement with Krishnamoorthy & Hinkle (1984) and O'Shea *et al.* (1984). Our values differ somewhat from

Fig. 1. Measurement of valinomycin-induced $\Delta\psi$ and initial rate of proton influx

Mitochondrial protein (4 mg) was incubated in 4 ml of 200 mM-sucrose, 20 mM-LiCl, 2 mM-MgCl₂, 1 mM-Tris/HCl, 0.5 mM-EGTA, 5 μM -TPMP bromide, 5 μM -rotenone, 0.23 μM -myxothiazol, 0.82 μg of oligomycin/ml, 33 μM -*N*-ethylmaleimide, pH 7.00, at 25 °C, for 3 min. The $\Delta\psi$ induced by the addition of 0.5 μg of valinomycin (Val.) was followed with a TPMP⁺ electrode (upper trace), calibrated by additions of 2.5 nmol of TPMP up to 5 μM -TPMP. The initial rate of proton uptake (lower trace) was simultaneously followed with a pH electrode, calibrated by additions of 20 nmol of HCl.

Fig. 2. Passive proton flux versus membrane potential with variable external KCl

The data were derived as in Fig. 1 but in the presence of 0–1 mM-KCl. The maximal reverse pH gradient (before valinomycin, with zero added K⁺), measured by methylamine accumulation was 0.43 (± 0.04) pH units.

Fig. 3. Steady state respiration rate versus membrane potential, titrated with malonate

Mitochondria were incubated as in Fig. 1 but in the absence of valinomycin and myxothiazol, and the presence of 2 mM-succinate (lithium salt) and 0–1.5 mM-malonate (lithium salt). Steady state oxygen consumption and membrane potential were followed simultaneously with oxygen and TPMP⁺ electrodes. The maximal ΔpH (in the absence of malonate) measured by acetate accumulation was $0.27(\pm 0.07)$ pH units and decreased roughly in proportion with $\Delta\psi$.

Fig. 4. Respiration rate (●) and proton flux rate (□) versus membrane potential

The data are derived from Figs. 2 and 3, and replotted on a logarithmic scale.

those of the above authors due to the fact we have measured $\Delta\psi$, rather than inferring it from the presumed K^+ distribution.

With the same mitochondrial preparation and virtually the same conditions and medium, we also measured the steady state respiration rate on succinate as a function of $\Delta\psi$ titrated with the respiratory inhibitor malonate. Fig. 3 shows the usual non-ohmic rise in respiration rate with $\Delta\psi$. In Fig. 4, the data from Figs. 2 and 3 are replotted together on a logarithmic scale. The graph shows that the rise in both the proton flux rate and the steady state respiration rate is roughly exponential with

$\Delta\psi$ (although some other functions are not excluded), and further the two graphs can be superimposed for a H^+/O stoichiometry of 5. We infer that the respiration-versus- $\Delta\psi$ titration of Fig. 3 can be entirely explained by the passive proton permeability characteristics of the mitochondria, and Fig. 4 would tend to exclude the possibility of a H^+/O change over the range of $\Delta\psi$ used. The most generally accepted H^+/O stoichiometry from succinate to oxygen is 6 (Brand, 1977), or possibly 8 (Costa *et al.*, 1984). The stoichiometry of 5 which best fits the data in Fig. 4 is likely to be an underestimate due to the presence of a ΔpH of maximally 16 mV in the malonate titration, and the reversed ΔpH present during the proton flux measurements.

The role of ΔpH in proton permeability

To assess whether the ΔpH component of Δp contributes to the non-ohmic proton permeability of mitochondria, we titrated the respiration rate with malonate in the presence of valinomycin and external K^+ , in order to fix the $\Delta\psi$ component of Δp at a low value. In these conditions, assuming a fixed H^+/O stoichiometry, we can assess the effect of ΔpH on proton permeability. Fig. 5 shows a malonate titration of the steady state respiration rate, with $\Delta\psi$ fixed at about 90 mV with valinomycin and 2 mM external K^+ . From this it would appear that ΔpH has a strong effect on proton permeability. However under these conditions the mitochondria are swollen due to K^+ uptake, and may be partially damaged; therefore the titration was repeated at a lower external K^+ concentration (0.2 mM added K^+). Fig. 6 shows that under these conditions ΔpH still has considerable control over proton permeability, which is similar per mV to flux control by $\Delta\psi$. To be sure that the determinations at low respiration rate had attained a true steady state for protons, a time course of measured

Fig. 5. Malonate titration of respiration rate and ΔpH at fixed membrane potential (with valinomycin and 2 mM added K^+)

Mitochondrial protein (3 mg) was incubated in 1 ml of 180 mM-sucrose, 20 mM-LiCl, 5 mM-Hepes, 2 mM-KCl, 2 mM-succinate (lithium salt), 1 mM-EGTA, 10 μM -potassium acetate, 5 μM -rotenone, 5 μM -TPMP bromide, 2 μg of oligomycin/ml, 1 μg of valinomycin/ml, pH 7.0, at 25 °C, for 2 min. Malonate (lithium salt, pH 7.0) (0, 0.5, 1.5 or 4.0 mM final concn.) was then added and the oxygen consumption rate measured after a further 3 min, or the mitochondria were centrifuged with isotopes to measure matrix volume, $\Delta\psi$, and ΔpH . $\Delta\psi$ (●), ΔpH (■), and Δp (▲) are plotted against respiration rate.

Fig. 6. Malonate titration of respiration and ΔpH at fixed membrane potential (with valinomycin and 0.2 mM added K^+)

Incubation was as in Fig. 5, except 0.2 mM-KCl replaced 2 mM-KCl, and titration was with 0, 1, 2.5, or 5.5 mM of malonate (lithium salt).

respiration rate, $\Delta\psi$ and ΔpH was performed from 2 to 20 min after addition of 2.5 mM-malonate. There was no change in any of these parameters over the time course and therefore we concluded that the proton flux had reached a true steady state after the 3 min normally used in determinations. To check that the radioactive probes of $\Delta\psi$ and ΔpH registered a Δp close to zero at zero respiration rate, respiration was inhibited with excess myxothiazol (0.67 nmol/mg of protein), and Δp was measured to be 17 mV; however, estimates of ΔpH using [^3H]acetate are rather inaccurate under these conditions due to the reversed ΔpH and consequent low matrix concentration of acetate.

The interpretation of Figs. 5 and 6 might be complicated if the endogenous K^+/H^+ exchanger is induced in these conditions (Bernardi & Azzone, 1983), which in combination with valinomycin might lead to K^+ cycling and proton entry. To circumvent this possibility we used a second, slightly different, approach to investigate the role of ΔpH on proton permeability. Mitochondria were allowed to attain a steady state respiring on succinate in the absence of valinomycin, then nigericin (a K^+/H^+ exchange ionophore) was added to change the relative contribution of $\Delta\psi$ and ΔpH to the

steady state Δp . This resulted in a slight increase in respiration rate; therefore a small amount of malonate was also added to return the respiration rate to roughly the original level. Table 1 shows the averages from six such experiments. The addition of nigericin plus a small amount of malonate resulted in a drop in ΔpH of about 15 mV and a rise in $\Delta\psi$ of 15 mV, with little change in respiration rate, indicating that $\Delta\psi$ and ΔpH have roughly equivalent effects (per mV) on the passive proton permeability (assuming a constant H^+/O stoichiometry).

Cation permeability at high $\Delta\psi$

Brierley (1978), on the basis of an investigation of cation permeability in heart mitochondria, suggested that electrophoretic cation permeability may be voltage gated. This suggested to us that mitochondrial cation permeability might rise with $\Delta\psi$ in a similar way to proton permeability. Measurement of initial rates of swelling in acetate or phosphate salts of cations presents a simple method of assessing changes in permeability, as the rate of absorbance decrease (from the same initial absorbance) is proportional to the rates of salt entry (Massari *et al.*, 1972), and in acetate salts the cation uniport activity is normally rate-limiting (Brierley, 1976). Thus, to assess relative K^+ uniport activity as a function of $\Delta\psi$ we monitored the initial rate of swelling of mitochondria suspended in 120 mM- K^+ acetate, upon addition of succinate. $\Delta\psi$ was manipulated by including limiting amounts of either malonate or the uncoupler FCCP. The mitochondria were preincubated in the medium, before addition of succinate, and there was no swelling or shrinkage before succinate addition. Addition of valinomycin stimulated the rate of swelling by at least 5-fold in all conditions, indicating that K^+ uniport activity was always rate limiting for swelling. $\Delta\psi$ was monitored with a TPMP $^+$ -sensitive electrode.

Fig. 7 shows the initial rate of swelling on addition of succinate plotted against $\Delta\psi$, which was lowered with malonate or FCCP. K^+ uniport activity increased dramatically at high $\Delta\psi$, in a similar fashion to proton permeability. Similar curves were obtained by comparing rates of swelling at any time up to at least 3 min after succinate addition, so that the relationship is not restricted to the initial energization phase. The fact that the FCCP titration and malonate titration give similar curves shows that the swelling was not limited by proton permeability or electron transport rate themselves, but rather by $\Delta\psi$.

Table 1. Changes in respiration rate, ΔpH , and $\Delta\psi$ in static head on addition of nigericin and malonate

Mitochondrial protein (12 mg) was incubated in 3 ml of 100 mM-KCl, 5 mM-sucrose, 5 mM-Hepes, 5 mM-succinate (K^+ salt), 2 mM-MgCl $_2$, 1 mM-EGTA, 10 μ M-potassium acetate, 5 μ M-TPMP bromide, 5 μ M-rotenone, 3 μ g of oligomycin/ml, pH 7.0, at 25 $^{\circ}$ C, and after 3 min 1.5 μ g of nigericin was added, and after a further 2 min 9 μ l of 0.5 mM-malonate (K^+ salt) was added. Oxygen consumption rates were determined at 3, 5, and 7 min, and in parallel, in the presence of isotopes, mitochondria were centrifuged to determine matrix volume, ΔpH , and $\Delta\psi$. The data are the means of six separate experiments, in each of which each determination was made in at least triplicate.

Additions	Respiration rate (nmol of O/min per mg)	ΔpH (mV)	$\Delta\psi$ (mV)	Δp (mV)
None	10.2	21.6	138.7	160.3
+ Nigericin	12.6	7.6	155.3	162.9
+ Malonate	9.4	6.6	153.4	160.0

Fig. 7. Rate of swelling in potassium acetate versus membrane potential

Mitochondrial protein (6 mg) was incubated in 3 ml of 120 mM-potassium acetate, 5 mM-Hepes, 1 mM-EGTA, 10 μ M-TPMP bromide, 5 μ M-rotenone, 1 μ g of oligomycin/ml, pH 7.1, in the presence of 0–3 mM-malonate (potassium salt), or 0–100 pmol of FCCP/mg of protein for 2 min, and the initial rate of absorbance decrease was followed at 540 nm, after the addition of 15 μ l of 0.5 mM-succinate (potassium salt, pH 7.1). ▲, static head; ●, malonate titration; ■, FCCP titration.

Fig. 8. Rate of swelling in TMA acetate versus membrane potential

Mitochondria (3 mg of protein) were incubated in 3 ml of 120 mM-TMA acetate, 5 mM-succinate (TMA salt), 5 mM-Hepes, 1 mM-EGTA, 5 μ M-rotenone, 1 μ g of oligomycin/ml, pH 7.1, at 25 °C, in the presence of 0–3 mM-malonate (TMA salt), or 0–67 pmol of FCCP/mg of protein, and the initial rate of absorbance decrease was followed at 540 nm, after the addition of 15 μ l of 0.5 mM-succinate (TMA salt, pH 7.1). ▲, static head; ●, malonate titration; ■, FCCP titration.

The rates of swelling in the acetate salts of TMA (Fig. 8) and choline (Fig. 9) were also investigated. The relationship between swelling rate and $\Delta\psi$ was similar to that for K⁺ acetate, indicating a large increase in TMA and choline permeability above 150 mV. This suggests that there is a non-specific increase in cation permeability at high $\Delta\psi$.

Fig. 9. Rate of swelling in choline acetate versus membrane potential

Mitochondria (3 mg of protein) were incubated in 3 ml of 120 mM-choline acetate, 5 mM-succinate (choline salt), 5 mM-Hepes, 1 mM-EGTA, 5 μ M-rotenone, 1 μ g of oligomycin/ml, pH 7.1, at 25 °C, in the presence of 0–5 mM-malonate (choline salt), or 0–67 pmol of FCCP/mg of protein, and the initial rate of absorbance decrease was followed at 540 nm, after the addition of 15 μ l of 0.5 mM-succinate (choline salt, pH 7.1). ▲, static head; ●, malonate titration; ■, FCCP titration.

DISCUSSION

Several explanations have been put forward for the shape of the electron transport rate versus Δp titrations of the type shown in Fig. 2. Nicholls (1974) first suggested that they might be explained by a non-ohmic increase in passive proton permeability of the inner mitochondrial membrane. Krishnamoorthy & Hinkle (1984) showed that the proton permeability did indeed rise non-ohmically with $\Delta\psi$. We show here that the respiratory titrations can be wholly and quantitatively accounted for by the proton permeability characteristics.

Pietrobon *et al.* (1981) suggested that the respiratory titrations could be almost wholly accounted for by a drop in H⁺/O stoichiometry of the respiratory chain ('redox slippage') at high Δp . However, a drop in H⁺/O stoichiometry of at least 10-fold from succinate to oxygen would be required to account for these authors' own titrations of respiration rate versus Δp . This is difficult to reconcile with current conceptions of the mechanism of the respiratory chain, and we have previously shown (Brown & Brand, 1985) from thermodynamic estimates of the H⁺/O stoichiometry of the cytochrome *bc*₁ complex that this complex still moves 2H⁺/2e⁻ vectorially in static head. On the other hand, it has been shown that inhibitor titrations of electron flux from succinate to ferricyanide (Pietrobon *et al.*, 1981) and of the ATPase during ATP hydrolysis (Pietrobon *et al.*, 1983) give rise to input flux rates versus Δp curves that significantly deviate from those found when titrating electron flux from succinate to oxygen. If these deviations cannot be accounted for by experimental differences, such as matrix

volumes or conditions, then there may be some role for changes in effective or intrinsic H^+ stoichiometry in explaining these results.

A third possible explanation of the respiratory titrations is a heterogeneity of coupling of the mitochondrial preparation, as suggested by Duszynski & Wojtczak (1985). This follows from two postulates: (a) a proportion of the mitochondria in an isolated preparation may be uncoupled relative to the rest of the population, and (b) the more uncoupled mitochondria may be more sensitive to respiratory inhibitors due to the respiratory chain having a higher control strength over electron flow in the uncoupled state. If these two postulates are true then addition of a small amount of respiratory inhibitor to a heterogeneous mitochondrial preparation will inhibit respiration mainly of the relatively uncoupled mitochondria, and since these mitochondria will have a relatively low Δp , initially the inhibition of respiration will be accompanied by only a small drop in Δp . This model would in principle explain titrations with malonate, as uncoupled respiration is much more sensitive to malonate than is coupled respiration (Pietrobon *et al.*, 1981), due to the fact that succinate dehydrogenase and succinate transport are rate-limiting in the uncoupled state. However it does not explain antimycin titrations of respiration on succinate, as the cytochrome bc_1 complex does not have a higher control strength over respiration in the uncoupled state. Pietrobon *et al.* (1981) found that for up to at least 50% inhibition of rate, the coupled rate (on a percentage basis) is not less (but in fact slightly more) sensitive to antimycin (and funiculosin) than the uncoupled rate, yet during the titration with low concentrations of antimycin (or funiculosin) of coupled mitochondria, the Δp versus respiration rate curves are just as non-linear as with malonate. This effectively falsifies the heterogeneity explanation.

A fourth possible explanation of the inhibitor titrations is given by the 'mosaic protonic coupling' hypothesis of Westerhoff *et al.* (1984). This predicts a non-ohmic relationship between electron transport rate and the bulk phase Δp even when ohmic flow-force relationships for proton movements are assumed. This is essentially a consequence of respiratory chain inhibitors also functionally inhibiting localized proton leaks within coupling units. However this type of model is not capable of explaining the non-ohmic relation between passive proton flux and the bulk phase $\Delta\psi$ (shown in Fig. 2), which by itself is capable of fully accounting for the inhibitor titrations.

That the relatively large respiration rate (and proton permeability) at static head is a function of Δp , rather than solely $\Delta\psi$ or ΔpH , was suggested by Nicholls (1977) on the experimental basis that the relative contributions of $\Delta\psi$ and ΔpH could be changed (with valinomycin and K^+) without causing much change in the static head Δp in coupled brown adipose tissue mitochondria. However, the contention only follows from this result if the static head respiration rate was also unchanged, but the rate was not measured (or shown). We show here that $\Delta\psi$ and ΔpH have roughly equivalent effects (per mV) on the steady state respiration rate, and thus presumably the passive proton permeability is a function of Δp rather than either of its components.

That the passive proton flux rises non-ohmically with $\Delta\psi$ is not in itself remarkable, as this has been found to be a general characteristic of carrier-mediated ion

transport in black lipid membranes (Eisenmann *et al.*, 1975). It has been suggested that the current-voltage curves can be explained by a putative charge carrier crossing a trapezoid energy barrier in the membrane (Krishnamoorthy & Hinkle, 1984). The passive proton permeability of the mitochondrial membrane shares several characteristics with the proton permeability of phospholipid bilayers, in that the permeability is large relative to other ions, and increases at high $\Delta\psi$ (Krishnamoorthy & Hinkle, 1984; Gutknecht, 1984); however, the proton permeability of the mitochondrial lipids alone has been estimated to be an order of magnitude lower than that of the intact mitochondrial membrane (Krishnamoorthy & Hinkle, 1984). Thus it is not clear whether the majority of the proton flux through the mitochondrial membrane passes via the proteins, or the lipids as modified by the presence of proteins, or through protein-lipid interfaces. The finding presented here that cation permeabilities (including unphysiological cations) greatly increase at high $\Delta\psi$, in a similar way to proton permeability, suggests that the mechanism leading to increased permeability is non-specific. Possible mechanisms might include endogenous lipids acting as ionophores at high $\Delta\psi$, or some form of physical perturbation of the membrane, such as dielectric breakdown. Electrical breakdown of planar bilayers occurs at $\Delta\psi$ of between 100 and 300 mV (Thompson & Henn, 1970; El-Mashak & Tsong, 1985).

The consequence of a greatly increased permeability at high Δp for mitochondrial function *in vivo* are manifold and important. If the normal Δp *in vivo* is large enough, the passive proton permeability characteristics will buffer Δp against any other change in proton flux, and this would help to buffer the cytosolic phosphorylation potential, through both kinetic and thermodynamic effects on phosphorylation. Also, hormonal stimulation of mitochondrial dehydrogenases or changes in substrate type might be thermogenic. However, it has not as yet been shown that mitochondria *in vivo* have the same proton permeability characteristics or that the Δp *in vivo* is large enough to give rise to a significant passive proton flux.

The permeability characteristics might also provide a teleological rationale for why the H^+ stoichiometries of the redox pumps and ATPase are so high. The high H^+ stoichiometries of these pumps results in a relatively low equilibrium Δp , which enables the mitochondria to avoid the large inefficiency and heat production (and perhaps dielectric breakdown), that would result from a high equilibrium Δp .

Respiration-dependent swelling in acetate salts of cations is thought to reflect an energy-dependent electrophoretic cation uniport (Brierley, 1976). In heart mitochondria the cation uptake is unspecific, supporting swelling in acetate salts of K^+ , Li^+ , $Tris^+$, TMA^+ , and Mg^{2+} at rates proportional to the coupled respiration rate (Brierley *et al.*, 1971). $^{42}K^+$ influx rates show similar properties and are inhibited during phosphorylation (Jung *et al.*, 1977). From these and other results Brierley has suggested that the energy dependent cation uniport activity may be non-carrier-mediated (Brierley, 1976) and 'voltage gated' (Brierley, 1978). Hansford & Lehninger (1972) have also shown an energy-dependent increase in unspecific cation permeability in blowfly flight-muscle mitochondria, which is inhibited during phosphorylation. Our results reported here support the idea of a 'voltage

gated', unspecific cation permeability in mitochondria. This would explain why cation uptake is inhibited by phosphorylation, as $\Delta\psi$ drops by about 50 mV during phosphorylation (Nicholls, 1974), enough to suppress cation uptake greatly. A voltage-gated swelling in K^+ phosphate might also help to explain the well-known ultrastructural transitions of mitochondria that accompany energization and state 4–3 transitions (Hackenbrock, 1972). Energization to a state-4 Δp would lead to swelling in K^+ or Na^+ phosphate media, resulting in the swollen 'orthodox' configuration, while the drop in $\Delta\psi$ accompanying state-3 conditions would inhibit swelling and allow a constant endogenous K^+/H^+ or Na^+/H^+ exchange activity to cause shrinkage to the 'condensed' configuration. This is consistent with the finding that the ultrastructural transitions are dependent on K^+ phosphate movements (Webster & Bronk, 1978).

G.C.B. is grateful to the Medical Research Council for the award of a research studentship.

REFERENCES

- Bernardi, P. & Azzone, G. F. (1983) *Biochim. Biophys. Acta* **724**, 212–223
- Brand, M. D. (1977) *Biochem. Soc. Trans.* **5**, 1615–1620
- Brierley, G. P. (1976) *Mol. Cell. Biochem.* **10**, 41–62
- Brierley, G. P. (1978) in *The Molecular Biology of Membranes* (Fleischer, S., Hatefi, Y., MacLennan, D. H. & Tzagoloff, A., eds.), pp. 295–308, Plenum Press, New York
- Brierley, G. P., Jurkowitz, M., Scott, K. M. & Merola, A. J. (1971) *Arch. Biochem. Biophys.* **147**, 545–546
- Brown, G. C. & Brand, M. D. (1985) *Biochem. J.* **225**, 399–405
- Chappell, J. B. & Hansford, R. G. (1972) in *Subcellular Components: Preparation and Fractionation* (Birnie, G. D., ed.), 2nd edn., pp. 77–91, Butterworths, London
- Clark, A. J., Cotton, N. P. J. & Jackson, J. B. (1983) *Eur. J. Biochem.* **130**, 575–580
- Costa, L. E., Reynafarje, B. & Lehninger, A. L. (1984) *J. Biol. Chem.* **259**, 4802–4811
- Cotton, N. P. J., Clark, A. J. & Jackson, J. B. (1984) *Eur. J. Biochem.* **142**, 193–198
- Duszynski, J. & Wojtaz, L. (1985) *FEBS Lett.* **182**, 243–248
- Eisenmann, G., Krasne, S. & Ciani, S. (1975) *Ann. N.Y. Acad. Sci.* **264**, 34–59
- El-Mashak, E. M. & Tsong, T. Y. (1985) *Biochemistry* **24**, 2884–2888
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Gutknecht, J. (1984) *J. Membr. Biol.* **82**, 105–112
- Hackenbrock, C. R. (1972) *J. Cell. Biol.* **53**, 450–465
- Hansford, R. G. & Lehninger, A. L. (1972) *Biochem. J.* **126**, 689–700
- Jung, D. W., Chavez, E. & Brierley, G. P. (1977) *Arch. Biochem. Biophys.* **183**, 452–459
- Kell, D. B., John, P. & Ferguson, S. J. (1978) *Biochem. Soc. Trans.* **6**, 1292–1295
- Krishnamoorthy, G. & Hinkle, P. C. (1984) *Biochemistry* **23**, 1640–1645
- Mandolino, G., Desantis, A. & Melandri, B. A. (1983) *Biochim. Biophys. Acta* **723**, 428–439
- Massari, S., Frigeri, L. & Azzone, G. F. (1972) *J. Membr. Biol.* **9**, 57–70
- Nicholls, D. G. (1974) *Eur. J. Biochem.* **50**, 305–315
- Nicholls, D. G. (1977) *Eur. J. Biochem.* **77**, 349–356
- O'Shea, P. S., Petrone, G., Casey, R. P. & Azzi, A. (1984) *Biochem. J.* **219**, 719–726
- Pietrobon, D., Azzone, G. F. & Walz, D. (1981) *Eur. J. Biochem.* **117**, 389–394
- Pietrobon, D., Zoratti, M. & Azzone, G. F. (1983) *Biochim. Biophys. Acta* **723**, 317–321
- Schonfeld, M. & Neumann, J. (1977) *FEBS Lett.* **73**, 51–54
- Sorgato, M. C. & Ferguson, S. J. (1979) *Biochemistry* **18**, 5737–5742
- Thompson, T. E. & Henn, F. A. (1970) in *Membranes of Mitochondria and Chloroplasts* (Racker, E., ed.), Van Nostrand Reinhold Co., New York
- Webster, K. A. & Bronk, J. R. (1978) *J. Bioenerg.* **10**, 23–44
- Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F. & Kell, D. B. (1984) *Biochim. Biophys. Acta* **768**, 257–292

Received 23 August 1985/24 September 1985; accepted 22 October 1985