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Non-Ohmic Proton Conductance of Mitochondria and Liposomes†

G. Krishnamoorthy and Peter C. Hinkle*

ABSTRACT: Direct measurements of the proton/hydroxyl ion flux across rat liver mitochondria and liposome membranes are reported. H^+/OH^- fluxes driven by membrane potential ($\Delta\psi$) showed nonlinear dependence on $\Delta\psi$ both in mitochondria and in liposomes whereas ΔpH-driven H^+/OH^- flux shows linear dependence on ΔpH in liposomes. In the presence of low concentrations of a protonophore the H^+/OH^- flux was linearly dependent on $\Delta\psi$ and showed complex dependence on ΔpH. The nonlinearity of H^+/OH^- permeability without protonophore is described by an integrated Nernst–Plank equation with trapezoidal energy barrier. Permeability coefficients depended on the driving force but were in the range 10^{-3} cm/s for mitochondria and 10^{-4}–10^{-6} cm/s for liposomes. The nonlinear dependence of H^+/OH^- flux on $\Delta\psi$ explains the nonlinear dependence of electrochemical proton gradient on the rate of electron transport in energy coupling systems.

It is generally accepted that the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$ across the mitochondrial inner membrane is the intermediate which couples respiration to ATP synthesis (Mitchell, 1961, 1979). However, a complete quantitative description of proton fluxes in this system has proven difficult and controversial. The measured coupling ratios for proton transport by the respiratory chain differ by as much as 3-fold (Hinkle, 1981). Even the usual assumption that there is an invariant coupling ratio has been challenged, and the concept of variable stoichiometry and molecular slipping of proton pumps has been proposed (Rottenberg, 1979a; Walz, 1979; Westerhoff et al., 1981; Pietrobon et al., 1981). An important factor in these determinations which has not been well studied is the kinetics of proton permeability across the mitochondrial inner membrane. Direct measurements of proton permeability were made by Mitchell & Moyle (1967). An indirect method was devised by Nicholls (1974), who measured the magnitude of $\Delta\tilde{\mu}_{H^+}$ formed by respiration when the respiration rate was partially inhibited with malonate. If it is assumed that the coupling ratio of proton transport by the respiratory chain is constant, then this analysis gives the relative proton permeability as a function of $\Delta\tilde{\mu}_{H^+}$. The result was a nonlinear curve with increasing permeability coefficient at high values of $\Delta\tilde{\mu}_{H^+}$. Similar observations have been made with submitochondrial particles (Sorgato & Ferguson, 1979) and bacteria (Kell et al., 1978; Jackson, 1982; Clark et al., 1983). An alternative interpretation of Nicholl's results proposed by Pietrobon et al. (1981, 1983) is that the coupling ratio of respiration-driven proton transport decreased at high values of $\Delta\tilde{\mu}_{H^+}$ and the proton permeability coefficient is constant. To distinguish between these two interpretations, we have directly measured the equivalent proton flux, J_{H^+/OH^-}, driven by membrane potential, $\Delta\psi$, and pH gradient, ΔpH, in rat liver mitochondria and liposomes.

Materials and Methods

Rat liver mitochondria were prepared according to Pedersen et al. (1978) in 0.25 M sucrose. Respiratory control ratios of 6–10 were obtained with succinate as substrate. Myxothiazol was a generous gift from Dr. W. Trowitzsch (Gesellschaft für Biotechnologische Forschung, Braunschweig).

Potassium-loaded sonicated liposomes (for the measurement of $\Delta\psi$-induced proton flux) were made from lipids derived from either soybean [acetone-washed asolectin (Kagawa & Racker, 1971)] or beef heart mitochondria (Kagawa et al., 1973). Lipids were suspended in a medium containing from 135 to 500 mM KCl and 0.5 mM MOPS[1] at pH 7.1 (with 10% w/w potassium cholate in the case of mitochondrial lipids) at a concentration of 50–70 mg/mL and sonicated for about 30 min until clear. The pH was readjusted to 7.1, and the external K^+ was removed by passage through a (30 × 1.5 cm) Sephadex G-50 column equilibrated with a medium containing 230 mM sucrose, 20 mM LiCl, 0.5 mM LiMOPS, and 0.4 mM Li_2EGTA at 4 °C.

Liposomes for the measurement of ΔpH-driven proton flux were prepared by sonicating the lipids in 100 mM potassium citrate, pH 5.0, followed by passage through the Sephadex column equilibrated with 0.1 mM MES, pH 5.0. Imposition of ΔpH and the measurement of proton flux are described in the legend to Figure 6.

Proton flux measurements were carried out in a well stirred and thermostated chamber fitted with a combination pH and K^+-sensitive electrodes connected to electrometers and a dual channel recorder. The half-times of response for both electrodes were less than 1 s. The rates of change in the external

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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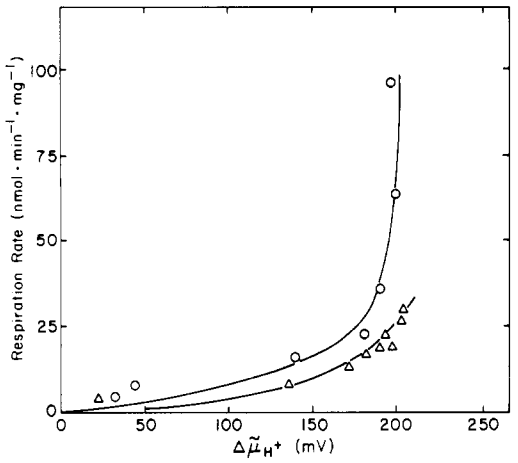

FIGURE 1: Dependence of $\Delta\tilde{\mu}_{H^+}$ on the rate of respiration in rat liver mitochondria. $\Delta\tilde{\mu}_{H^+}$ was measured after steady-state respiration proceeded for 4 min. The medium contained 240 mM sucrose, 10 mM NaMOPS, 20 mM glucose, 0.5 mM KCl, 1.5 mM NaP_i, 5 mM $MgCl_2$, 0.25 mg/mL bovine serum albumin, 0.5 μM valinomycin, 8 μM rotenone, 30 μM ^{86}Rb, 50 μM [^{14}C]acetate, [^{3}H]sucrose, and 1 mg/mL mitochondria at pH 7.2. When succinate was substrate (△), 5 mM succinate and 0–3 mM malonate were present. When ascorbate was substrate (○), 5 mM sodium ascorbate, 0.1 μg/mL antimycin A, and 0.4 μM–0.4 mM TMPD were present.

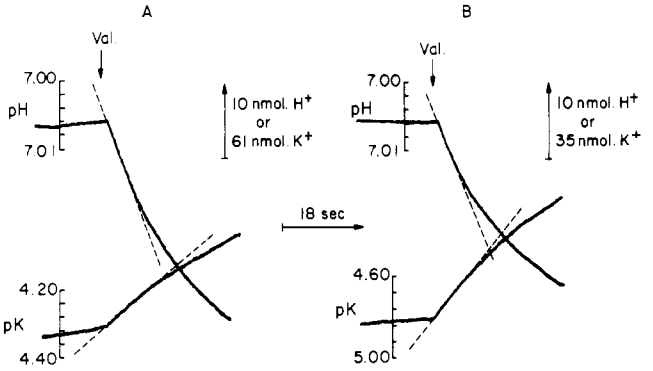

FIGURE 2: Recordings of H^+ and K^+ in experiments designed to measure $\Delta\psi$-driven J_{H^+/OH^-} in rat liver mitochondria (A) and mitochondrial lipid liposomes (B). Mitochondria were suspended at a protein concentration of 1.4 mg/mL in a medium containing 230 mM sucrose, 20 mM LiCl, 0.5 mM LiMOPS, 0.4 mM Li_2EGTA, and variable concentrations (0.01–30 mM) of KCl at pH 7.1. After the addition of mitochondria, the following inhibitors were added: rotenone (20 μM), myxothiazol (1.4 μM), oligomycin (1.2 μg/mL), and *N*-ethylmaleimide (100 μM). Following equilibration at 25 °C for 3 min, proton influx was initiated by the addition of valiomycin (0.08 μg/mL final concentration). In the case of liposomes (B) the inhibitors were not included, and valinomycin was added to a final concentration of 0.4 μg/mL.

pH were converted into H^+/OH^- flux values by using the external buffering power.

The membrane potential created by the K^+ gradient when valinomycin is added was calculated from the Nernst equation:

$$\Delta\psi = 59 \log ([K^+]_{in}/[K^+]_{out}) \quad (mV)$$

The internal K^+ concentration of mitochondria was assumed to be 135 mM (Pietrobon et al., 1982). The internal K^+ concentration of liposomes was assumed to be the concentration during sonication. Leakage, swelling, or shrinkage may have changed the internal K^+ concentration in some experiments, which would result in a shift of all the calculated values by a small amount (18 mV for a 2-fold change).

$\Delta\tilde{\mu}_{H^+}$ was measured by the method of Nicholls (1974) using ^{86}Rb uptake in the presence of valinomycin to measure $\Delta\psi$ and [^{14}C]acetate uptake to measure ΔpH with separation of the mitochondria from the medium by filtration without washing with correction for external volume on the filter using [^{3}H]-sucrose as a volume label. The reaction was run for 4 min in an oxygen electrode to measure respiration, and then the same sample was filtered to measure probe uptake. Internal volumes of mitochondria were measured by centrifugation through silicone oil in media containing [^{14}C]sucrose and [^{3}H]glycerol as described by Rottenberg (1979b). The internal volumes measured ranged from 1.0 to 1.3 μL/mg of protein.

Results

Dependence of $\Delta\tilde{\mu}_{H^+}$ on Respiration Rate. The relationship between the respiration rate and $\Delta\tilde{\mu}_{H^+}$ in rat liver mitochondria measured by the method of Nicholls (1974) is shown in Figure 1. The nonlinearity is more pronounced in the case of respiration with ascorbate (titrated with the mediator TMPD) than with succinate (titrated with malonate). Such nonlinear respiration rate–$\Delta\tilde{\mu}_{H^+}$ curves have been reported by many workers (Nicholls, 1974; Sorgato & Ferguson, 1979; Pietrobon et al., 1981). The curve with ascorbate–TMPD as substrate is above that with succinate as substrate because the number of protons transported electrogenically by electron transfer from TMPD to oxygen is two less than from succinate to oxygen (cf. Hinkle, 1981).

$\Delta\psi$-Driven H^+/OH^- Flux. Figure 2 shows recordings of external H^+ and K^+ in typical experiments designed to measure $\Delta\psi$-driven H^+/OH^- flux, J_{H^+/OH^-}, in rat liver mitochondria and liposomes made from lipids extracted from bovine heart mitochondria. The observed pH changes are expressed as H^+/OH^- fluxes because it is impossible to distinguish between H^+ flux in one direction and OH^- flux in the other. Valinomycin-induced K^+-diffusion potential was varied by varying the K^+ concentration in the suspending medium. J_{H^+/OH^-} was measured from the initial rate of proton uptake. Measurement of initial rates of proton uptake and K^+ efflux gave J_{H^+}/J_{K^+} ratios in the range 0.5–0.7 for mitochondria and 0.3–0.7 for liposomes, indicating that protons (or hydroxyl ions) are a significant fraction of the ions moving in response to the imposed $\Delta\psi$. However, this ratio did not vary within a single batch of mitochondria or liposomes used in these experiments. In the case of mitochondria the medium contained *N*-ethylmaleimide (NEM), oligomycin, and EGTA (in addition to respiratory chain inhibitors) to avoid proton movement through the P_i/OH^- antiporter, F_0–F_1-ATPase, and Ca^{2+}/H^+ antiporter, respectively. Omission of EGTA caused a more rapid initial K^+ efflux, presumably because of electrogenic Ca^{2+} uptake, but no change in the H^+ uptake curve (not shown). Omission of NEM or oligomycin caused an increase in the initial rate of H^+ influx, leading to a strongly biphasic uptake curve (not shown). The fact that only the initial rate of H^+/OH^- flux is used in our analysis also avoids possible electroneutral pathways by contaminating permeant acids or bases.

The dependence of J_{H^+/OH^-} on $\Delta\psi$ is shown in Figure 3 for mitochondria and in Figures 4 and 5 for liposomes. In the absence of protonophore, J_{H^+/OH^-} shows non-ohmic behavior, with J_{H^+/OH^-} increasing more rapidly than $\Delta\psi$ in all the systems. That the valinomycin induced K^+ movement was not limiting the observed J_{H^+/OH^-} was confirmed by titration with valinomycin as shown in the insets to Figures 3–5. Note that the dependence of J_{H^+/OH^-} on $\Delta\psi$ becomes linear in the presence of protonophore both in mitochondria and in liposomes, consistent with the observations of Jackson (1982) and Clark et al. (1983) on the carotenoid band-shift kinetics in *Rhodopseudomonas capsulata*. The uptake of protons would

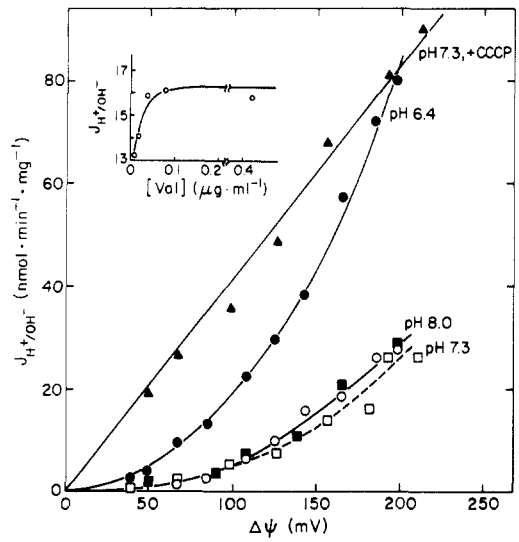

FIGURE 3: Dependence of J_{H^+/OH^-} on $\Delta\psi$ in mitochondria. The points are at pH 7.3 in the presence (▲) and in the absence (□ and ■) of protonophore CCCP (4.2 nM) and at pH 6.4 (●) and pH 8.0 (○) in the absence of protonophore. The inset shows valinomycin concentration dependence of J_{H^+/OH^-} at $\Delta\psi$ = 182 mV and pH 7.3. For experimental details see the legend of Figure 2 and Materials and Methods. The flux refers to mg^{-1} of mitochondrial protein.

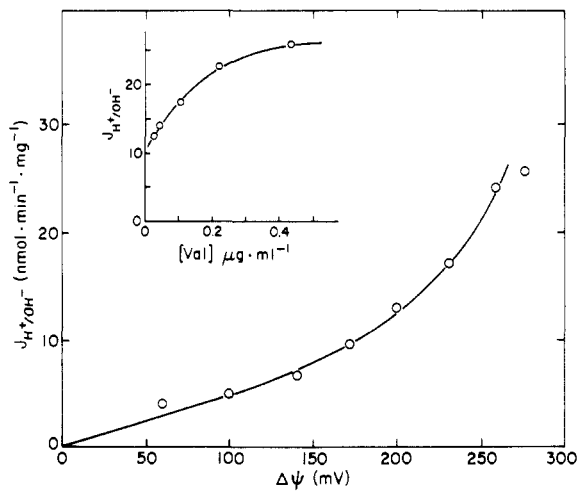

FIGURE 4: Dependence of J_{H^+/OH^-} on $\Delta\psi$ in mitochondrial lipid liposomes at pH 7.1 in the absence of protonophore. The inset shows valinomycin dependence of J_{H^+/OH^-} at $\Delta\psi$ = 259 mV. The flux refers to mg^{-1} of lipid.

result in the formation of ΔpH with polarity opposite to that of the imposed $\Delta\psi$ (acid inside). This could result in overestimation of the driving force, $\Delta\tilde{\mu}_{H^+}$. However, this is unlikely to affect our measurements of initial rates especially in the case of mitochondria where the internal buffering power of 14 nmol of H^+·mg^{-1}·(pH unit)$^{-1}$ (Mitchell & Moyle, 1969) would limit the reverse ΔpH during the period of flux measurement to less than 0.3 pH unit (18 mV).

The data in Figure 3 show that in mitochondria J_{H^+/OH^-} values are very similar at pH 7.3 and 8.0 whereas those at pH 6.4 are higher by a factor of ~2.5. Comparison of the data in Figures 4 and 5 shows that $\Delta\psi$-driven H^+/OH^- flux is 3-fold higher in liposomes of mitochondrial lipids than in soybean lipids, suggesting higher H^+/OH^- permeability for mitochondrial lipid liposomes. This difference could arise from the different lipid compositions but is also partly caused by the use of cholate in formation of liposomes from the mitochondrial lipids. Use of cholate with asolectin caused a 2-fold increase in proton permeability (not shown). For comparison with other studies H^+/OH^- conductance (g_{H^+/OH^-}) values

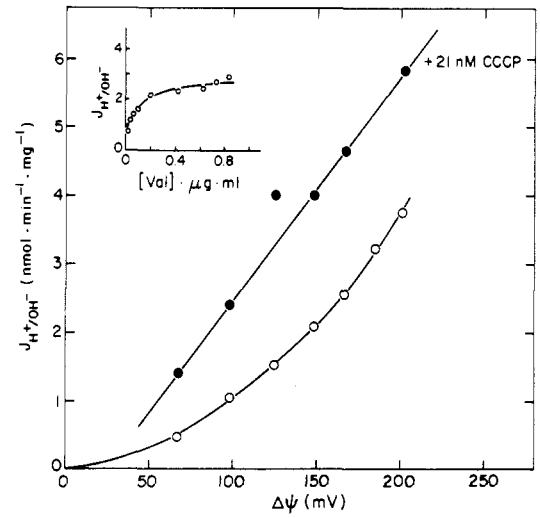

FIGURE 5: Dependence of J_{H^+/OH^-} on $\Delta\psi$ in soybean lipid vesicles at pH 7.1 in the presence (●) and in the absence (○) of protonophore CCCP (21 nM). The inset shows valinomycin concentration dependence of J_{H^+/OH^-} at $\Delta\psi$ = 172 mV. The flux refers to mg^{-1} of lipid.

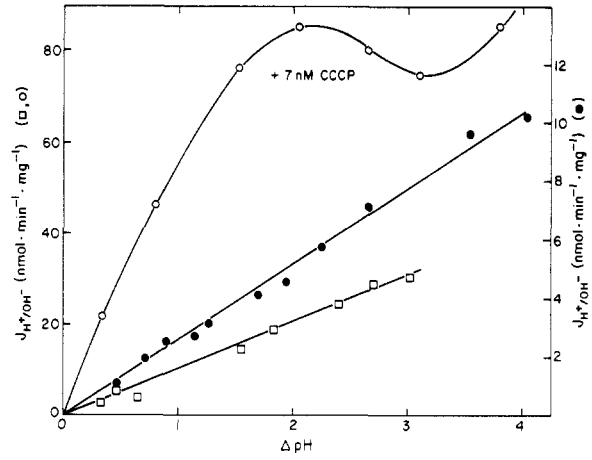

FIGURE 6: Dependence of J_{H^+/OH^-} on ΔpH in lipid vesicles. A 2.1-mL sample of a medium containing 175 mM KCl, 0.5 mM MES, 0.5 mM HEPES, and 0.5 mM CHES and pH varying in the range 5–9.5 was taken in a thermostated (25 °C) well-stirred vessel fitted with a combination pH electrode. About 0.2 mL of liposomes containing 100 mM potassium citrate, pH 5.0, inside and 175 mM KCl and 0.1 mM MES outside was added to the medium creating a ΔpH, and the initial rate of external pH change was recorded. J_{H^+/OH^-} was calculated from the initial slopes of pH recording. The points are mitochondrial lipid liposome in the absence of protonophore (□), mitochondrial lipid liposome in the presence of 7 nM CCCP (○), and asolectin liposome in the absence of protonophore (●). Left scale is for mitochondrial lipid liposomes, and right scale is for asolectin liposomes.

calculated from H^+/OH^- flux at $\Delta\psi$ = 150 mV at pH near 7 were 0.36 μS·cm^{-2} for mitochondria and 0.02 and 0.007 μS·cm^{-2} for liposomes of mitochondrial or soybean lipids, respectively. These values were calculated by assuming a surface area of 4 × 10^2 cm^2·(mg of protein)$^{-1}$ for mitochondria (Mitchell & Moyle, 1967) and 4 × 10^3 cm^2·(mg of lipid)$^{-1}$ (70 Å^2 per lipid molecule) for liposomes (Mimms et al., 1981). Mitchell & Moyle (1967) reported a value of 0.45 μS·cm^{-2} for mitochondria at pH 7.2 calculated from ΔpH-driven H^+/OH^- flux and found it to be independent of ΔpH in a narrow range of ΔpH (~0.5 pH unit).

ΔpH-Driven H^+/OH^- Flux. In contrast to $\Delta\psi$-driven H^+/OH^- flux, ΔpH-driven H^+/OH^- flux (Figure 6) showed linear dependence on ΔpH over a wide range of ΔpH in liposomes in the absence of protonophore. In the presence of the protonophore CCCP the dependence became complex. In

these experiments valinomycin was present to ensure that H^+/OH^- flux is not limited by counterion current. J_{H^+/OH^-} values were about 4-fold higher in mitochondrial lipid liposomes than in asolectin liposomes as observed for $\Delta\psi$-driven flux. J_{H^+/OH^-} was insensitive to the presence of valinomycin in the case of asolectin vesicles whereas it was lower by about 2-fold in the absence of valinomycin in mitochondrial lipid liposomes (not shown). The H^+/OH^- conductances calculated from the data shown in Figure 6 are 0.066 and 0.016 $\mu S \cdot cm^{-2}$ for mitochondrial lipid and asolectin liposomes, respectively.

Analysis of Results

$\Delta\psi$-Driven H^+/OH^- Flux. Current–voltage (I–V) characteristics of carrier-mediated ion transport in black lipid membranes have been analyzed by several workers (Walz et al., 1969; Neumke & Laüger, 1969; Laüger & Stark, 1970; Stark & Benz, 1971; Stark et al., 1971; Haydon & Hladky, 1972; Hall et al., 1973; Hladky, 1974; Benz & McLaughlin, 1983). In general, I–V curves have been found to be nonlinear, with the conductance increasing with increasing voltage. According to the general consensus in the field, the nonlinearity arises due to the image force barrier (Neumke & Laüger, 1969) in the membrane acting on charged species. Hall et al. (1973) noted that a trapezoidal energy barrier is a good model for the image force barrier. Hladky (1974) showed that for a trapezoidal barrier the current I depends on the applied voltage according to

$$I = G_0 bu \sinh(u/2)/\sinh(bu/2) \quad (1)$$

with $u = F\Delta\psi/(RT)$ where F is the Faraday constant. G_0 is the conductance at zero voltage, and b is the fraction of the membrane thickness spanned by the flat top of the trapezoid. $b = 1$ would lead to a linear I–V relationship whereas $b = 0$ would represent a single-jump Eyring model.

In order to analyze our $\Delta\psi$-driven H^+/OH^- flux (in the absence of uncoupler) in terms of this model, we have plotted $J_{H^+/OH^-}/J^{125}_{H^+/OH^-}$ vs. $\Delta\psi$ as in Figure 7. $J^{125}_{H^+/OH^-}$ is the value of the flux at $\Delta\psi = 125$ mV. The lines in Figure 7 are calculated by using the equation

$$\frac{J_{H^+/OH^-}}{J^{125}_{H^+/OH^-}} = \frac{u}{u^{125}} \times \frac{\sinh(u/2)/\sinh(bu/2)}{\sinh(u^{125}/2)/\sinh(bu^{125}/2)} \quad (2)$$

It can be seen that $b = 0.6$ and $b = 0.75$ fit the experimental data in mitochondria and liposomes, respectively. Evaluation of b relies mainly on the data at high values of $\Delta\psi$.

The linear dependence of J_{H^+/OH^-} on $\Delta\psi$ in the presence of protonophore (Figures 3 and 5) may seem to be in contradiction to observations of others (Benz & McLaughlin, 1983). These authors showed that conductance by FCCP obeys eq 1 with $b = 0.65$. However, our results are steady-state measurements on proton flux whereas Benz & McLaughlin (1983) measured initial instantaneous ionic conductance. The effective proton flux J_{H^+/OH^-} consists of both H^+ and OH^- ions. If both have the same value of b, then the analysis (Figure 7) is still valid, but the fraction of the flux carried by each ion cannot be determined. However, in this analysis we simply wish to stress that H^+/OH^- fluxes are nonlinear with respect to $\Delta\psi$ and that there is a reasonable theoretical basis for this.

Evaluation of the H^+/OH^- net permeability coefficient, P_{H^+/OH^-}, from the data in Figures 3–5 does not give a unique value because of the non-ohmic behavior of $\Delta\psi$-driven H^+/OH^- conductance. However, the values of P_{H^+/OH^-} calculated at $\Delta\psi = 150$ mV are 1.6×10^{-3} $cm \cdot s^{-1}$ for mitochondria and 5.2×10^{-5} and 1.9×10^{-5} $cm \cdot s^{-1}$ for liposomes of mitochondrial and soybean lipids, respectively, at pH near 7.

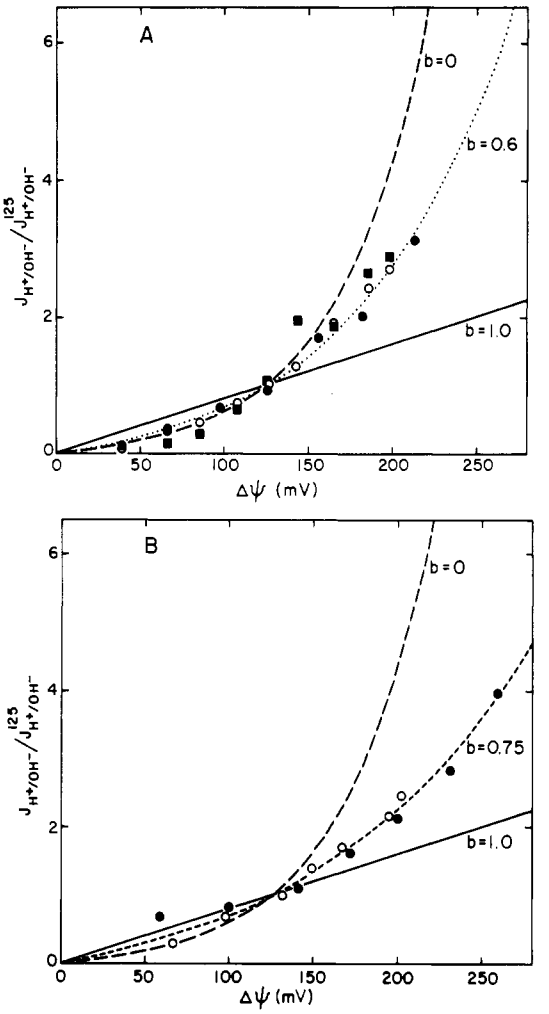

FIGURE 7: Plots of $\Delta\psi$ dependence of flux ratios. In panel A the data with mitochondria from Figure 3 at pH 6.4 (○), 7.3 (●), and 8.0 (■) are plotted. In panel B the data with liposomes from Figure 4 (●) and Figure 5 (○) are plotted. The lines are theoretical curves simulated by using eq 2.

ΔpH-Driven H^+/OH^- Flux. Several workers [for example, see Nichols & Deamer (1980), Nozaki & Tanford (1981), and Rossignol et al. (1982)] have used the diffusion equation

$$J_{H^+/OH^-} = P_{H^+}([H^+]_i - [H^+]_o) + P_{OH^-}([OH^-]_o - [OH^-]_i) \quad (3)$$

to describe the net flow of H^+ and OH^- across liposome membranes. P_{H^+} and P_{OH^-} are the individual permeability coefficients for H^+ and OH^-, respectively. Our data (Figure 6), which show linear dependence of J_{H^+/OH^-} on ΔpH, cannot be fitted by using eq 3 for any choice of pH-independent values of P_{H^+} and P_{OH^-}. However, we have calculated P_{H^+/OH^-} using eq 3 by using the data with ΔpH <1.5. They are $\sim 1 \times 10^{-4}$ $cm \cdot s^{-1}$ for asolectin and $\sim 4 \times 10^{-4}$ $cm \cdot s^{-1}$ for mitochondrial lipid liposomes.

Mitchell & Moyle (1967) used an equation of the type

$$J_{H^+/OH^-} = (P_{H^+}[H^+]_m + P_{OH^-}[OH^-]_m)\Delta pH \quad (4)$$

to describe ΔpH-driven H^+/OH^- flux in mitochondria. $[H^+]_m$ and $[OH^-]_m$ are the concentrations of H^+ and OH^- in the membrane phase. Mitchell and Moyle have observed linear dependence of J_{H^+/OH^-} on ΔpH in a small pH range around pH 7. Inspection of eq 4 shows that even this equation would not predict a linear relationship of J_{H^+/OH^-} and ΔpH unless the terms in parentheses are independent of ΔpH. The linearity observed in our experiments (Figure 6), where the in-

ternal pH is kept constant at pH 5 and the external pH is varied from 5 to 9.5, might indicate that either $P_{OH^-} \ll P_{H^+}$ or $[H^+]_m$ and $[OH^-]_m$ are not significantly pH dependent. Even these assumptions would not allow us to calculate P_{H^+} since we cannot assume a value for $[H^+]_m$. However, we can calculate H^+/OH^- conductance, g_{H^+/OH^-}, from our data on ΔpH-driven flux taking 1 ΔpH unit to be 59 mV. They are 0.066 and 0.016 $\mu S \cdot cm^{-2}$ for mitochondrial lipid and asolectin liposomes, respectively.

Another equation relating H^+/OH^- flux to ΔpH has been found experimentally in chloroplasts by Davenport (1983). They have measured indirectly H^+/OH^- flux from the rate of coupled flux of electrons and found the following relationship:

$$J_{H^+/OH^-} = K([H^+]_i/[H^+]_o) \quad (5)$$

However, our data (Figure 6) cannot be fitted with such an equation.

Discussion

The analysis of our results shows that a trapezoidal image force barrier can explain the nonlinear H^+/OH^- flux driven by $\Delta\psi$ in mitochondria and liposomes. ΔpH-driven H^+/OH^- flux is directly proportional to ΔpH rather than obeying Fick's diffusion equation. The integrated Nernst–Planck equation does not fit our data on ΔpH-driven H^+/OH^- flux, and we have not found a general equation describing the H^+/OH^- flux driven by both $\Delta\psi$ and ΔpH. The values of the net permeability coefficient P_{H^+/OH^-}, which depend on the driving force, are in the range 1×10^{-3} $cm \cdot s^{-1}$ for mitochondria and 10^{-4}–10^{-6} $cm \cdot s^{-1}$ for liposomes. These are close to the values reported by Deamer & Nichols (1983).

Measurements of relative H^+/OH^- permeability of mitochondria by indirect methods (Figure 1) similar to those of Nicholls (1974) and Sorgato & Ferguson (1979) also show a nonlinear increase in the rate at high values of $\Delta\tilde{\mu}_{H^+}$. This is consistent with the direct measurements of H^+/OH^- flux driven by $\Delta\psi$ because $\Delta\tilde{\mu}_{H^+}$ was predominantly $\Delta\psi$. In Figure 1 the curve with succinate as substrate is similar in shape to that of the directly measured H^+/OH^- permeability. The curve with ascorbate plus TMPD as substrate was much steeper at high values of $\Delta\tilde{\mu}_{H^+}$, however, which may be caused by a possible alternative pathway of electron transfer in cytochrome oxidase (Wikström & Penttilä, 1982), cyclic electron transfer in the cytochrome *b* region with TMPD, or some other proton-conducting activity related to the presence of artificial redox mediators. It has been proposed that proton pumps of the respiratory chain may "slip", allowing electron transfer without proton transport or proton permeability without electron transport (Pietrobon et al., 1981, 1983). The agreement between the nonlinearity of H^+/OH^- permeability in mitochondria and liposomes and indirect measurements with succinate as substrate indicate that the simple permeability of the lipid bilayer can account for the results without envoking the "slippage" of proton pumps or other protein-mediated pathways of H^+/OH^- permeability in normal mitochondria. The roughly 10-fold higher H^+/OH^- permeability of mitochondria compared to that of liposomes of equal area could be an indirect effect of proteins which increase the dielectric constant of the membrane and thus increase proton flux through the lipids [see Dilger & McLaughlin (1979)], or it could be direct proton conductance by some proteins. Brown adipose tissue mitochondria contain a 32K protein which conducts H^+ or OH^- across the membrane for heat production (Nicholls, 1977). Portis et al. (1975) and Davenport & McCarty (1983) measured indirectly a nonlinear H^+/OH^- conductance in chloroplasts which was inhibited by ATP or DCCD, indicating that it was an activity of the CF_1–F_0-ATPase. The presence of oligomycin in our experiments should eliminate possible conduction by F_1–F_0-ATPase or by the high H^+/OH^- conductance of F_0 after dissociation of F_1.

The nonlinear H^+/OH^- permeability of mitochondria is also relevant to general chemiosmotic coupling reactions and accounts for the well-known phenomenon of "loose coupling" where mitochondria which have no (or low) respiratory control can still synthesize ATP. This occurs because at high $\Delta\tilde{\mu}_{H^+}$ the H^+/OH^- leak and respiration are high, but when ADP is added, $\Delta\tilde{\mu}_{H^+}$ decreases, and the H^+/OH^- leak is greatly reduced, allowing flux of H^+ through the F_1–F_0-ATPase to synthesize ATP at the same respiration rate. The H^+/OH^- leak is also important in calculating the efficiency of oxidative phosphorylation. Recent measurements have indicated that after correction for H^+/OH^- permeability, the theoretical P/O ratio for synthesis of external ATP by mitochondria is 1.5 with succinate as substrate (Hinkle & Yu, 1979; Berry & Hinkle, 1983).

Registry No. CCCP, 555-60-2; H^+, 12408-02-5; K, 7440-09-7; OH^-, 14280-30-9.

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Pathway for Uncoupler-Induced Calcium Efflux in Rat Liver Mitochondria: Inhibition by Ruthenium Red†

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ABSTRACT: The rate of uncoupler-induced Ca^{2+} efflux from rat liver mitochondria is increased by acetate and decreased by phosphate. This effect depends on a shift of the apparent K_m, which is increased by phosphate and decreased by acetate, while the V_{max} is not modified. The modification of the apparent K_m by permeant anions presumably reflects changes in the concentration of matrix free Ca^{2+}. A major part of uncoupler-induced Ca^{2+} efflux is sensitive to Ruthenium Red, the specific inhibitor of the Ca^{2+} uniporter, but an apparent insensitivity is observed when the H^+ permeability is rate limiting in the process of Ca^{2+} efflux. The titer of uncoupler required for maximal stimulation of Ca^{2+} efflux increases with the Ca^{2+} load and may be 1–2 orders of magnitude higher than that required for maximal stimulation of respiration. On the other hand, when the uncoupler concentration is raised above 10^{-6} M, the process of Ca^{2+} efflux becomes again Ruthenium Red insensitive. The Ruthenium Red inhibition of uncoupler-induced Ca^{2+} efflux is time dependent, in that the degree of inhibition exerted by low amounts of Ruthenium Red increases with the incubation time. Since the inhibition of the rate of Ca^{2+} influx undergoes a parallel relief, it is possible that Ruthenium Red moves from the cytosolic to the matrix side of the inner membrane. It is concluded that, in native mitochondria, uncoupler-induced Ca^{2+} efflux occurs via reversal of the uniport Ca^{2+} carrier, and not through activation of an independent pathway.

The inhibition of Ca^{2+} transport by lanthanides (Mela, 1968, 1969; Vainio et al., 1970; Scarpa & Azzone, 1970; Reed & Bygrave, 1974; Lehninger et al., 1978) and Ruthenium Red (Moore, 1971; Vasington et al., 1972) has been taken as evidence that Ca^{2+} transport across the inner mitochondrial membrane occurs through a specific carrier [for recent reviews, see Saris & Akerman (1980) and Nicholls & Akerman (1982)]. The inhibition is competitive for La^{3+} (Scarpa & Azzone, 1970; Reed & Bygrave, 1974) and noncompetitive for Ruthenium Red (Reed & Bygrave, 1974) and Pr^{3+} (Mela, 1969). Due to the specificity of its inhibitory effect, Ruthenium Red has become an important tool to define the pathways for Ca^{2+} transport. Addition of Ruthenium Red to aerobic mitochondria, where Ca^{2+} uptake has reached a steady state, results in Ca^{2+} release (Puskin et al., 1976; Pozzan et al., 1977). This has been interpreted as evidence for the existence of two independent pathways for Ca^{2+} transport (Carafoli, 1979; Nicholls & Crompton, 1980): the Ruthenium Red sensitive, electrophoretic pathway for Ca^{2+} uptake and the Ruthenium Red insensitive, presumably electroneutral pathway for Ca^{2+} efflux, activated by Na^+ in excitable tissues [Crompton et al., 1978; for reviews, see Nicholls & Akerman (1982)] and by high $\Delta\psi$[1] in liver (Bernardi & Azzone, 1982, 1983). Besides the Ruthenium Red sensitivity and the nature of the driving force, the two pathways differ significantly in the kinetics, since the rate of Ruthenium Red sensitive Ca^{2+} uptake is 2–3 orders of magnitude higher than the Ruthenium Red insensitive Ca^{2+} efflux.

In 1972, Vasington et al. observed that the very rapid Ca^{2+} release induced by uncouplers is not inhibited by Ruthenium Red. This observation has been repeatedly confirmed in subsequent studies [Puskin et al., 1976; Pozzan & Azzone, 1977; Pozzan et al., 1977; Zoccarato & Nicholls, 1982; cf., however, Sottocasa et al. (1977), Luthra & Olson (1977), and Rigoni et al. (1980)] and attributed to a release of Ruthenium

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[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Mops, 4-morpholinepropanesulfonic acid; $\Delta\psi$, membrane potential; Tris, tris(hydroxymethyl)aminomethane.

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