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The Proton Permeability of Liposomes Made from Mitochondrial Inner Membrane Phospholipids: Comparison with Isolated Mitochondria

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Abstract. Unilamellar liposomes with native phospholipid fatty acid composition were prepared from rat liver mitochondrial inner membrane phospholipids by extrusion in medium containing 50 mM potassium. They were diluted into low potassium medium to establish a transmembrane potassium gradient. A known membrane potential was imposed by addition of valinomycin, and proton flux into liposomes was measured. Valinomycin in the range 10 pM–1nM was sufficient to fully establish membrane potential. Valinomycin concentrations above 3 nM catalyzed additional proton flux and were avoided. At 300 pM valinomycin, proton flux depended nonlinearly on membrane potential. At 160 mV membrane potential the flux was 30 nmol H^+/min/mg phospholipid—approximately 5% of the proton leak flux under comparable conditions in isolated mitochondria, indicating that leak pathways through bulk phospholipid bilayer account for only a small proportion of total mitochondrial proton leak.

Key words: Mitochondria — Vesicle — Proton leak — Phospholipid — Liposome — Fatty acid

Introduction

Protons are pumped out across the mitochondrial inner membrane during electron transport, usually returning via the ATP synthase to make ATP. Alternatively, they may passively reenter the matrix—a physiologically important process accounting for about 25% of the respiration rate of resting hepatocytes [6] and 20–30% of the basal metabolic rate of a rat [44]. Except in brown adipose tissue [33] the mechanism of this proton leak is not known. There is no sound evidence for the involvement of specific proteins and the mechanism of proton leak is unknown [11].

It is unclear what proportion of mitochondrial proton leak flux can be explained by passive diffusion across the phospholipid bilayer portion of the inner membrane. Fuks and Homble [18] proposed that proton leak across planar bilayers made from thylakoid galactolipids can account for all of the proton permeability of thylakoid membranes. Mitchell and Moyle [31] were the first to directly measure proton conductance in mitochondria, reporting a value of 0.45 $\mu S/cm^2$. Krishnamoorthy and Hinkle [27] reported a mitochondrial proton conductance of 0.36 $\mu S/cm^2$, but a 20-fold lower liposomal value. O'Shea et al. [35] reported a 2.7-fold greater proton leak in mitochondria than in liposomes. A review of earlier proton permeability comparisons in biological and model membranes is provided by [16].

O'Shea et al. [35] compared soy-bean phospholipid liposomes with rat liver mitochondria, while Krishnamoorthy & Hinkle [27] compared soy-bean or beef heart total mitochondrial phospholipid liposomes with rat liver mitochondria. The mitochondrial inner membrane has a unique complement of phospholipids and fatty acids that may confer properties not shared by soy-bean or outer membrane phospholipids; the comparison should be performed using inner membrane phospholipids.

Previous investigations have used nonpolar solvents (especially n-decane) or detergents (especially cholate) to dissolve phospholipids prior to planar bilayer or liposome formation respectively [9, 27, 35]. Detergents (especially digitonin) are sometimes used in mitochondrial inner membrane preparation methods to solubilize the outer membrane. Detergents disrupt biological membranes and are acknowledged to alter proton leak in liposomes [27]. Since removal of all detergent or solvent can never be guaranteed, their use may lead to misestimation of native proton leak fluxes. Moreover, the

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long dialysis times required to remove detergents from liposome preparations have been shown to cause changes in patterns of phospholipid fatty acid unsaturation [9], thus detergent-based systems are best avoided for the current investigation.

In the work reported here, the problems described above were avoided to allow a more valid estimate of the contribution of proton leak through bulk phospholipid bilayer to mitochondrial proton leak. The proton permeability of isolated rat liver mitochondria was measured. The outer membrane was then removed by mechanical disruption and the inner membrane purified by centrifugation on discontinuous sucrose density gradients. Inner membrane phospholipids were isolated and formed into unilamellar liposomes by nondetergent methods. The proton permeability of these liposomes was assayed at 37°C using a better technique than previously employed [10], and compared to that of intact mitochondria.

Materials and Methods

Materials

Organic solvents were of analytical grade from BDH, Poole, Dorset, UK, except n-Hexane (GC grade). Hyflo-Supercel was from BDH. Standard fatty acids for gas chromatography were from Matreya, Pleasant Gap, PA. All other biochemicals were of reagent grade from Sigma, Poole, Dorset, UK. Rats were fed *ad libitum* on Special Diet Services economy rat maintenance diet (Lillico, Bletchworth Surrey UK); water was freely available.

Isolation of Mitochondrial Inner Membranes

Liver mitochondria were prepared from male Wistar rats (250 g) by established methods [43] in 250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, pH 7.4 at 4°C. Protein was assayed by the Biuret method [20]. Some mitochondria were set aside for proton leak measurements.

Mitochondrial inner membranes (mitoplasts) were isolated essentially by the method of Holland and Stevenson [23], with the addition of 1 mM EGTA per 125 mM sucrose to all media. This amount of EGTA was essential to avoid later problems with liposome formation. Mitochondria were swollen by suspending them at 20–40 mg protein/ml in 3 volumes of 10 mM Tris-phosphate, pH 7.4 at 4°C for 5 min, mixing gently. The suspension was then bought to a multiple of 16 ml by adding 1 volume of 1.8 M sucrose, 2 mM $MgSO_4$, 2 mM ATP, 14.4 mM EGTA, pH 7.4 at 4°C, and mixed gently for 5 min to shrink mitochondria.

Aliquots (16 ml) of the suspension were sonicated in a 25 ml glass beaker using an MSE 150 Soniprep set a 6 μm amplitude (19 mm probe) for 6 × 20 sec with 20 sec cooling on ice, then transferred to 40 ml ultracentrifuge tubes. Twenty ml of 1.18 M sucrose 9.44 mM EGTA pH 7.4 at 4°C was layered beneath each sonicate. Tubes were topped up with 250 mM sucrose and ultracentrifuged at 30,000 × *g* in a swing-out rotor for 3 hr, at 4°C.

Supernatants were removed. Pellets were washed and resuspended in ''lipid backwash medium'' (*see* phospholipid extraction, below) and homogenized. Assays of succinate cytochrome c reductase [47] and monoamine oxidase [48] (inner and outer membrane marker enzymes respectively) showed the inner membrane pellet contained 16 ± 3% (mean ± SEM, n = 6) of total outer membrane activity, and 80 ± 2% (mean ± SEM, n = 3) of total inner membrane activity.

Measurement of Mitochondrial Proton Leak

Mitochondrial proton leak was assayed by simultaneous measurement of respiration rate and membrane potential using oxygen and $TPMP^+$-sensitive electrodes [5]. Mitochondria were incubated at 1 mg protein/ml in 120 mM KCl, 5 mM HEPES, 1 mM EGTA, 0.3% (w/v) fat free BSA, pH 7.2 at 37°C. 4 mM succinate, 5 μM rotenone, 1 μg oligomycin/mg protein, 80 ng nigericin/ml, and 5 μM $TPMP^+$ were subsequently added. Respiration was inhibited by titrating in 1–10 mM potassium malonate. $TPMP^+$ binding was corrected for with a value of 0.45 previously obtained using protocol 7 of [5].

Phospholipid Extraction

All glassware was rinsed in 2% (v/v) acetic acid, 96% (v/v) ethanol and distilled de-ionized water. Total mitoplast lipids were extracted [17] in 10 volumes of 2:1 chloroform:methanol with 0.01% (w/v) butylated hydroxytoluene present as antioxidant. The protein ''cake'' was removed by qualitative paper filtration. The crude extract was washed with 2.5 volumes of ''lipid backwash medium'' containing 5 mM EDTA, 5 mM EGTA, 0.73% (w/v) NaCl, pH 7.4 at 20°C. The aqueous phase was extracted a further 3 times with 1 volume chloroform. Organic extracts were collected by filtration through anhydrous Na_2SO_4, dried under nitrogen stream and dispersed in 5 ml chloroform. A phosphorus assay was performed to determine the phospholipid content of the sample.

Phospholipids were isolated by silicic acid column chromatography [25] using 40 mg silicic acid (300 mesh, dried at 70°C for 3 hr) per mg phospholipid. Greater amounts of adsorbent made liposome formation more difficult, while less resulted in reduced sample recovery. A 2 mm layer of sand (washed in chloroform/methanol then dried) was layered over the adsorbent to protect the surface from disturbance. The total lipid sample was applied in chloroform, followed by 10 ml chloroform to elute nonpolar lipids. Phospholipids were eluted with 10 ml methanol, dried under nitrogen stream, and dispersed in 19:1 chloroform/methanol. They were then filtered through Hyflo-Supercel tightly packed between cotton wool plugs in a Pasteur pipette, to remove contaminating particulate silicic acid that otherwise adversely affected liposome formation. Phospholipids were stored in solvent under nitrogen at −80°C; under such conditions phospholipid fatty acid composition is stable for up to 12 weeks.

Preparation of Liposomes

Phospholipids were dried to a thin film in a round-bottomed flask under nitrogen stream then held under vacuum for 1 hr to remove residual solvent. Multilamellar liposomes were prepared by suspending phospholipids at 20–30 mg/ml in a medium containing 50 mM KCl, 100 mM HEPES, 1 mM EDTA, brought to pH 7.2 at 37°C with LiOH [3]. The medium was passed through a 100 nm filter and deoxygenated before use. Four glass beads (2 mm diameter) were added and the flask was flushed with nitrogen, sealed, and whirlimixed for 30-sec bursts with 30-sec rests at 37°C, until all phospholipid appeared to be in suspension. Suspension of phospholipids at concentrations above 30 mg/ml made subsequent extrusion difficult.

The suspension was frozen/thawed 5 times in a cryo-tube in liquid nitrogen/37°C water bath, then extruded 10 times at 600 psi of nitrogen through 2 stacked polycarbonate 100 nm pore filters (Costar, Cambridge, MA) in commercial apparatus (The Liposome Company, Van-

couver, BC) [24]. The 1.5 ml, 37°C-thermostatted chamber was flushed with nitrogen prior to each extrusion cycle to reduce lipid degradation. If the first cycle was slow (>1 min), blocked filters were replaced for remaining cycles. Resulting unilamellar liposomes were stored under nitrogen at 4°C until and during proton leak measurements. Negative stain electron micrographs (*not shown*) indicated the preparations were of uniform, vesicular nature.

Measurement of Liposomal Proton Leak

Liposomal proton leak was measured using a modified version of the method of Brand et al. [10]. 1.6 ml of proton leak medium (1 mM EDTA, 116.5 mM LiCl, brought to pH 7.2 at 37°C with LiOH) were incubated at 37°C in a 3-ml thermostatted glass chamber with pH and $TPMP^+$-sensitive electrodes. Following electrode equilibration (1–2 min), calibrating $TPMP^+$ was added stepwise to a final concentration of 0.5 μM. Liposomes (4 μl) were added, followed by 0.5 μl valinomycin in ethanol (final concentration 1 pM–300 nM). The resultant liposomal K^+ efflux established a $\Delta\Psi$ of 160 mV. Liposomal proton uptake was measured with a pH electrode, and $TPMP^+$ accumulation with a $TPMP^+$-sensitive electrode. At the end of the experiment, 0.5 μl of 0.2 mg Gramicidin D/ml in ethanol was added to collapse all ion gradients, and any small baseline drift was corrected for. The pH electrode was then calibrated with 5 μl of 1 mM HCl.

Liposomal $\Delta\Psi$ was altered by varying potassium concentration in the external medium or adding more/less liposomes, both of which change the transmembrane potassium gradient. The $TPMP^+$ electrode was used only semiquantitatively to check whether a full membrane potential was established and maintained during the experiment.

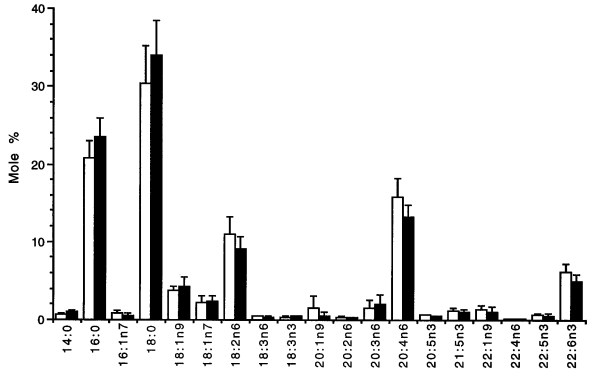
Estimation of $\Delta\Psi$ from $TPMP^+$ uptake assuming no $TPMP^+$ binding [32,42], as shown in Figs. 2 and 3, agreed (within 15 mV) with the imposed $\Delta\Psi$ of 160 mV. The estimation assumes a liposome trapped volume of 10% at 30 mg phospholipid/ml from calculations based on the geometry of liposomes (surface area: volume ratio of a sphere, surface area of a single phospholipid). When imposed $\Delta\Psi$ was varied (Fig. 4), $TPMP^+$ uptake varied accordingly, confirming that the $TPMP^+$ signal reflected liposomal $\Delta\Psi$. However, calculated potentials were about 30 mV higher than expected at an imposed $\Delta\Psi$ of 40 mV and about 20 mV lower than expected at an imposed $\Delta\Psi$ of 200 mV.

Phosphorus Assay

Total phosphorus was assayed by the method of Ames [1]. One ''ashing'' was found to be sufficient to release all phosphorus from samples. Phosphorus content was calculated using a standard curve constructed with KH_2PO_4. Phospholipid content was calculated assuming an average phospholipid molecular mass of 780.

Determination of Phospholipid Fatty Acid Composition

Phospholipid samples were derivatized to fatty acid methyl esters at 75°C in 20% (w/v) BF_3/methanol and purified by Florisil™ column chromatography [25]. Analysis was performed using a Hewlett Packard 5890 gas chromatograph with 30 m Supelcowax-10 capillary column (0.25 mm internal diameter, 0.25 μm coating) and a temperature program of 140–250°C at 3.5°/min with 20-min plateau, sufficient to elute all methyl esters. Injection (split ratio 100:1) and detection (flame ionization) were held at 260°C. Peaks were identified by their retention times relative to commercial standards. Mole % composition was calculated from integrated peak areas for fatty acids $C_{14:0}$ to $C_{22:6n3}$. Unidentified material in this region of the chromatogram was <5% of total.



Property	Mitochondria	Liposomes
Unsaturation index	152.3 ± 31.4	130.5 ± 26.2
Average chain length	18.3 ± 3.5	18.12 ± 3.12
% n3	8.9 ±1.9	7.6 ± 1.8
% n6	29.4 ± 5.8	25.2 ± 4.6
% n9	6.8 ± 2.5	6.0 ± 2.3
% saturates	51.9 ± 7.3	58.4 ± 7.2
% monounsaturates	9.9 ± 3.7	8.8 ± 3.4
% polyunsaturates	38.3 ± 7.6	32.8 ± 6.3

Fig. 1. Fatty acid composition of mitochondrial inner membrane phospholipids after isolation (□), and following reconstitution into liposomes (■), determined by gas chromatography as detailed in Materials and Methods. Values are expressed as relative mole %, and are means of six independent experiments ± SEM. Fatty acid nomenclature is [number of carbon atoms:number of double bonds *n* position of first double bond from the methyl end]. Unsaturation index is the number of double bonds per 100 fatty acid molecules. Average chain length is Σ(chain length × mole %) of individual species. There are no significant differences in composition between mitochondria and liposomes (Student's *t*-test, 95% confidence limits).

Results

Phospholipid Fatty Acid Composition

Figure 1 shows the fatty acid composition of mitochondrial inner membrane phospholipids after extraction and of the liposomes prepared from them. The inner membrane composition agrees with literature data for rat liver mitochondrial inner membrane phospholipids [14]. Liposomal fatty acid composition was virtually identical to this, hence our liposomes represent an adequate compositional model of the phospholipid portion of the mitochondrial inner membrane. Patterns of fatty acid unsaturation, which correlate with proton leak in whole mitochondria [8] were preserved in the liposomes. This contrasts with earlier work using detergent-dialyzed mitochondrial phospholipid liposomes [9], where extensive fatty acid degradation occurred during liposome preparation.

The fatty acid composition of the outer membrane fractions from the sucrose density gradients was very

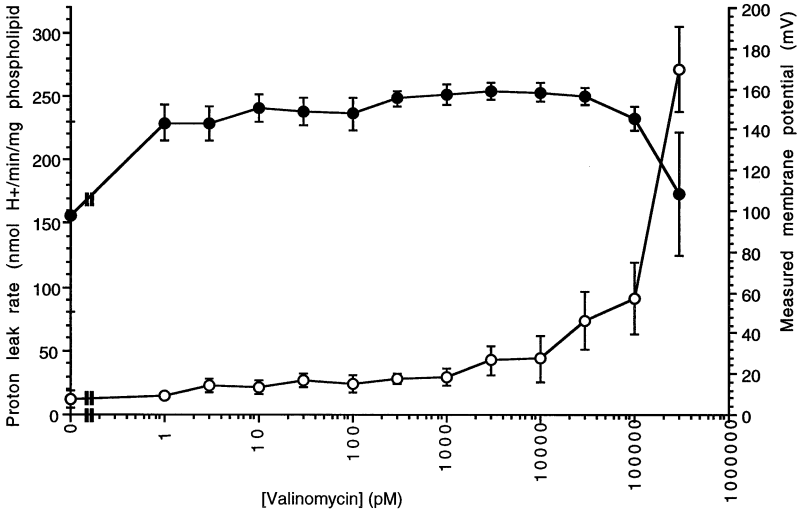


Fig. 2. Effect of valinomycin concentration on proton leak flux (○) and measured membrane potential (●) of mitochondrial inner membrane phospholipid liposomes prepared as described in Materials and Methods. $\Delta\Psi$ was set at 160 mV by the imposed K^+ gradient, established by the addition of valinomycin, and estimated from the $TPMP^+$ accumulation ratio. Results are means ± SEM, n = 4.

similar to the inner membrane composition (*results not shown*), confirming the observations of Colbeau, Nachbaur & Vignais [15].

Liposomal Proton Leak—Valinomycin Dependence

Figure 2 shows the proton leak and membrane potential (estimated from measured $TPMP^+$ uptake) of mitochondrial inner membrane phospholipid liposomes as a function of valinomycin concentration at an imposed $\Delta\Psi$ of 160 mV. In the range of 10 pM–1 nM valinomycin, $\Delta\Psi$ was fully established and proton leak flux was stable. Above 3 nM valinomycin, proton leak flux increased with no change in $\Delta\Psi$, indicating possible valinomycin protonophoric activity. Above 30 nM valinomycin, measured $\Delta\Psi$ decreased, possibly due to increased Li^+/K^+ exchange and partial collapse of the K^+ gradient. Valinomycin's secondary protonophoric activity saturated at 1.5–3 μM valinomycin with a proton leak of 520 ± 50 nmol H^+/min/mg phospholipid (*not shown*, mean ± SEM, $N = 4$). Valinomycin was used at 300 pM in subsequent experiments.

Garlid [19] reported protonophoric activity in liposomes at high valinomycin concentrations, and postulated formation of valinomycin:fatty acid ion pairs. We measured liposomal proton leak flux in the presence of fat-free BSA (*results not shown*). At 0.03% (w/v) BSA, with 2 μM valinomycin, proton leak fell by 63% relative to control. Ninety-one percent of the reduction was competed out by 12 μM palmitoyl-CoA, which itself does not increase proton leak flux, suggesting nearly all of the reduction is due to free fatty acid binding by BSA. At 0.05% (w/v) BSA, proton leak fell by 67%, but only 68% of the reduction was competed out by 24 μM palmitoyl-CoA, suggesting secondary effects of BSA such as protein:liposome surface interactions or valinomycin binding. However, at low (300 PM) valinomycin, proton leak flux is not significantly affected by 0.03% (w/v) BSA. These observations may support Garlid's hypothesis of a fatty acid:valinomycin ion pair mediated protonophoric activity, but only at higher valinomycin concentrations than those used in our experiments.

Previous studies have used higher valinomycin concentrations that may have contributed to the observed proton leak flux. Krishnamoorthy and Hinkle [27] found that in beef heart total mitochondrial phospholipid liposomes, valinomycin induced proton flux saturated at 360 nM valinomycin. Brand et al. [9] found that in rat liver total mitochondrial phospholipid liposomes, valinomycin saturation was complex, appearing to plateau at lower concentrations (500 nM) then rising further at much higher concentrations (2–3 μM). Some of these differences in valinomycin saturation might be explained by different experimental conditions, including the presence of detergents, methods of lipid isolation, and the type/composition of the lipids. No previous investigators measured liposomal $\Delta\Psi$, thus the reported values of proton leak may include secondary valinomycin effects. Nevertheless, it is clear from Fig. 1 that 300 pM valinomycin is sufficient to establish the full driving force for proton movement, without itself catalyzing additional proton flux.

Liposomal Proton Leak—CCCP Dependence

Figure 3 shows the proton leak of mitochondrial inner membrane phospholipid liposomes as a function of CCCP concentration at 300 pM valinomycin and an imposed $\Delta\Psi$ of 160 mV. Proton leak flux increased linearly with CCCP concentration, but $\Delta\Psi$ estimated from measured $TPMP^+$ uptake remained constant. This experiment indicates that our assay for proton leak is reliable.

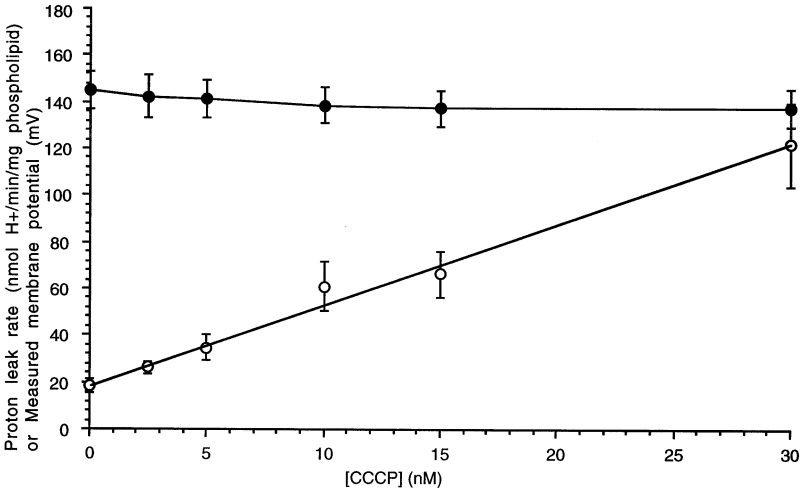


Fig. 3. Effect of CCCP concentration on proton leak flux (○) and measured membrane potential (●) of mitochondrial inner membrane phospholipid liposomes prepared as described in Materials and Methods. $\Delta\Psi$ was set at 160 mV by the imposed K^+ gradient, established by the addition of 300 pM valinomycin, and estimated from the $TPMP^+$ accumulation ratio. Results are means ± SEM, $n = 5$.

Liposomal Proton Leak—$\Delta\Psi$ Dependence

Figure 4 shows the proton leak of mitochondrial inner membrane phospholipid liposomes as a function of imposed membrane potential ($\Delta\Psi$) at 300 pM valinomycin. The bilayer proton leak flux was nonlinearly related to $\Delta\Psi$, as in mitochondria and reported in [27]. This differs from the linear relationship that has been reported between proton leak flux and ΔpH in liposomes [2,26,27]. The liposome proton leak at 160 mV $\Delta\Psi$ from many experiments of the kind shown in Figs. 2–4 was 29.6 ± 4.4 nmol H^+/min/mg phospholipid (mean ± SEM, n = 13). This equates to 0.110 $\mu S/cm^2$, compared with a previously reported value of 0.02 $\mu S/cm^2$ [27] for beef heart mitochondrial phospholipid liposomes at 150 mV, pH 7.

Mitochondrial Proton Leak

Figure 5 shows the $\Delta\Psi$ dependence of mitochondrial sate 4 (nonphosphorylating) respiration rate in the presence of 0.3% (w/v) fat-free BSA to chelate contaminating free fatty acids. These data are similar to previous findings [6,11] and give a value of 7.3 ± 0.2 nmol O/min/mg protein (mean ± SEM, $n = 3$) at 160 mV $\Delta\Psi$.

Discussion

We believe this study is one of the first to use a biological phospholipid liposome system that does not employ detergents or solvents, and maintains the native membrane fatty acid composition. Van de Vossenberg et al. studied liposomes from archaebacterial lipids [49], though they do not state whether lipid composition was affected during liposome formation. Applications of the methods outlined are many and varied.

Comparison of Liposomal and Mitochondrial Proton Leak

Proton leak flux in liposomes and mitochondria may be compared by converting to the same units. At 160 mV, liposome proton leak was 29.6 ± 4.4 nmol H^+/min/mg phospholipid. At the same $\Delta\Psi$, mitochondrial respiration rate was 7.3 ± 0.2 nmol O/min/mg protein. Using recoveries of 80% for the inner membrane preparation (marker enzyme assay), 95% for the total lipid extraction [17], and 87% for phospholipid extraction (phosphorus assay), we calculate that mitochondria contain 0.041 ± 0.003 mg inner membrane phospholipid per mg total mitochondrial protein. Using this figure, and a H^+/O stoichiometry of 6 for mitochondrial respiration with succinate as substrate [4], we arrive at a mitochondrial proton leak flux of 1073 ± 30 nmol H^+/min/mg inner membrane phospholipid. This value is 36-fold greater than the liposome rate.

Assuming that one phospholipid occupies a monolayer area of 80$Å^2$ (J.F. Nagle, *personal communication*) and that an average phospholipid has a molecular mass of 780, then 1 mg of phospholipid occupies 0.309 m^2 bilayer. Mitochondrial and liposome proton leak rates may thus be expressed as 3500 ± 100 and 96 ± 14 nmol $H^+/min/m^2$ phospholipid bilayer respectively.

Using data from the literature, other estimates of these figures may be derived. There is 0.056 m^2 inner membrane per mg mitochondrial protein (averaged values from [8,40,45]). Assuming the inner membrane to be 50% phospholipid bilayer by area [45], there is 0.091 mg inner membrane phospholipid per mg mitochondrial protein. Prebble [41] gives a value of 0.063 mg inner membrane phospholipid per mg mitochondrial protein. Substituting these values into the above calculation gives a mitochondrial proton leak per mg inner membrane phospholipid that is 16- or 23-fold greater than the liposome rate. We conclude that proton leak through bulk

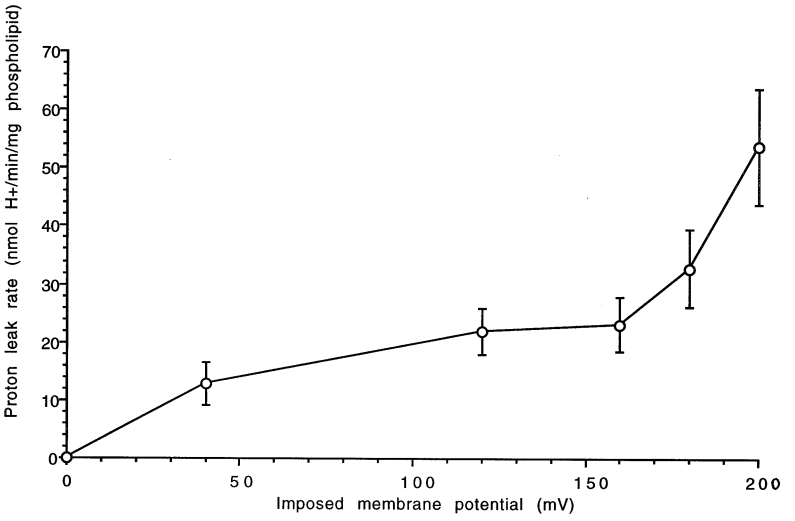


Fig. 4. Effect of imposed $\Delta\Psi$ on proton leak flux (○) of mitochondrial inner membrane phospholipid liposomes prepared as per Materials and Methods. $\Delta\Psi$ was varied by the imposed K^+ gradient and established by the addition of 300 pM valinomycin. Results are means ± SEM, $n = 4$.

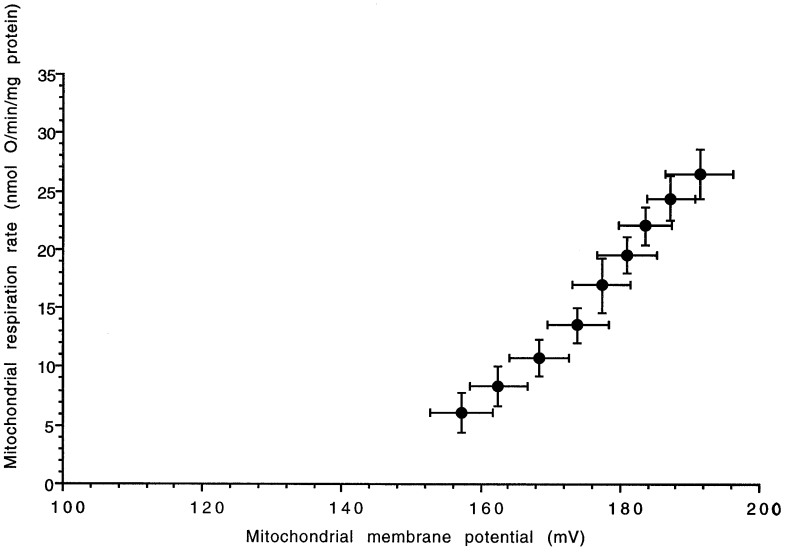


Fig. 5. Relationship between membrane potential and state 4 (nonphosphorylating) respiration rate (●) in isolated rat liver mitochondria respiring on succinate in the presence of 0.3% (w/v) BSA. Membrane potential was assayed with a $TPMP^+$-sensitive electrode, and respiration rate with an oxygen electrode, as described in Materials and Methods. Results are means ± SEM, $n = 3$.

phospholipid bilayer can account for only about 5% of the proton leak and state 4 (nonphosphorylating) respiration rate of isolated rat liver mitochondria. This agrees with the findings of Krishnamoorthy and Hinkle [27], despite major differences in methodology/techniques.

Possible Reasons for the Different Proton Leak of Liposomes and Mitochondria

How can we account for the difference in proton leak between mitochondria and liposomes prepared from them? One possible explanation is that the state 4 (nonphosphorylating) respiration rate of mitochondria is not caused by proton leak but by redox slip, in which less than six protons are pumped across the membrane per oxygen consumed (with succinate as substrate), at high membrane potential or electron transport rates [37]. However, redox slip is insignificant in isolated rat liver mitochondria at 37°C [7,13,39], and is not observed in isolated rat hepatocytes [39], so this does not appear to explain the difference.

Of the proposed mechanisms for proton leak in lipid bilayers, the favored model appears to be the water wire, in which a transmembrane hydrogen bonded chain of water molecules forms transiently, allowing protons to traverse [16]. It is thought that the gramicidin proton channel has a similar structure to a water wire, and current understanding of water wire mechanisms is based mainly on the gramicidin model [38]. Molecular dynamics simulations of such chains suggest that half-life (picoseconds) would give rise to rate constants for proton leak that match well to experimental values [29].

A second possibility is that the bilayer properties in

mitochondria differ from those in liposomes. This may favor the formation of water wires in mitochondria, accounting for their greater proton leak. For example, it is known that cardiolipin residues almost entirely in the outer leaflet of the mitochondrial inner membrane [22], and other as yet undiscovered lipid asymmetries may be present in mitochondria. In liposomes membrane asymmetry is presumably lost. The importance of asymmetry in determining proton permeability is unknown. Furthermore, the mitochondrial inner membrane is highly convoluted, whereas our 100 nm liposomes have little membrane curvature. It is unknown whether membrane curvature affects proton leak. The surface charge of bilayer phospholipids may be altered in mitochondria by the proximity of other parts of the inner or outer membrane, or by soluble proteins or ions, which may affect bilayer proton permeability [21]. Paula et al. [36] reported that proton permeability of liposomes increases disproportionately with bilayer thickness. The presence of proteins or certain lipid species in the native mitochondrial membrane which are absent from our liposomes may alter membrane thickness, and therefore, proton leak.

A third possibility is that transmembrane proteins may have nonspecific effects on membrane proton permeability. Since the mitochondrial inner membrane is approximately 50% protein by area [45], there is probably no phospholipid that is not influenced by nearby proteins, through their effects on packing, membrane dielectric, mobility and surface charge. It remains a strong possibility that nonspecific protein:lipid interactions allow the formation of proton conductance pathways that are absent from pure phospholipid liposomes [11]. However, Mimms et al. reported that incorporation of glycophorin-A into liposomes had no effect on cation permeabilities [30], and O'Shea et al. reported that incorporation of cytochrome oxidase into liposomes had no effect on proton permeability [35]. Observations in this laboratory (M.D. Brand and P.M. Stevenson, *unpublished*) showed that proton leak in mitochondrial phospholipid detergent-dialyzed liposomes was unaffected by incorporation of cytochrome P_{450} (detergent-free), but increased proportionately with incorporation of detergent-solubilized cytochrome oxidase. Oliver and Deamer recently reported that α-helical hydrophobic polypeptides form proton selective channels when incorporated into lipid bilayers [34]. They propose that the peptides may form aggregates with a central proton pore. However, it is unclear whether such aggregates could form in the mitochondrial inner membrane between the proteins of the respiratory chain.

A fourth possibility is that specific proteins or other membrane components absent from liposome preparations catalyze most of the mitochondrial proton leak. A candidate for such a role is the adenine nucleotide translocase, the most abundant inner membrane protein, particularly in view of its proposed involvement in mitochondrial fatty acid induced uncoupling [12] and its membership of the same protein superfamily as the uncoupling protein of brown adipose tissue [28]. However, the recent observation that twofold overexpression of the adenine nucleotide translocase in *Sacchromyces cerevisiae* has no effect on state 4 respiration of isolated yeast mitochondria [46] suggests that it does not catalyze significant endogenous mitochondrial proton leak. The involvement of other proteins and membrane components remains a possibility.

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