

# Incorporation of transmembrane hydroxide transport into the chemiosmotic theory

Aubrey D.N.J. de Grey \*

*Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK*

Received 27 May 1999; received in revised form 19 August 1999; accepted 17 September 1999

## Abstract

A cornerstone of textbook bioenergetics is that oxidative ATP synthesis in mitochondria requires, in normal conditions of internal and external pH, a potential difference ( $\Delta\psi$ ) of well over 100 mV between the aqueous compartments that the energy-transducing membrane separates. Measurements of  $\Delta\psi$  inferred from diffusion of membrane-permeant ions confirm this, but those using microelectrodes consistently find no such  $\Delta\psi$  — a result ostensibly irreconcilable with the chemiosmotic theory. Transmembrane hydroxide transport necessarily accompanies mitochondrial ATP synthesis, due to the action of several carrier proteins; this nullifies some of the proton transport by the respiratory chain. Here, it is proposed that these carriers' structure causes the path of this "lost" proton flow to include a component perpendicular to the membrane but within the aqueous phases, so maintaining a steady-state proton-motive force between the water at each membrane surface and in the adjacent bulk medium. The conflicting measurements of  $\Delta\psi$  are shown to be consistent with the response of this system to its chemical environment. © 1999 Elsevier Science S.A. All rights reserved.

**Keywords:** Oxidative phosphorylation; Proton circuit; Proton mobility; Membrane potential difference; Phosphate carrier; Hydroxide anion

## 1. Introduction

Mitchell's most comprehensive presentation of the chemiosmotic theory [1] noted explicitly that mitochondria do not maintain a difference of pH ( $\Delta\text{pH}$ ), between the two aqueous phases that their energy-transducing membrane separates, sufficient to drive the  $\text{F}_0\text{F}_1$ -ATPase backwards as the theory required. Since that membrane has low permeability not only to protons but also to other ions present in solution in vivo, Mitchell suggested that an unequal distribution of charge density at the two membrane surfaces — that is, a potential difference ( $\Delta\psi$ ) across the membrane — adds to the effect of  $\Delta\text{pH}$ , the two producing a sufficient "proton-motive force" (PMF, or  $\Delta p$ ) to drive ATP synthesis. Fig. 1 shows the various mitochondrial membrane transport and associated processes to be discussed in this article. The PMF  $\Delta p$  is related to the thermodynamically relevant quantity  $\Delta\tilde{\mu}_{\text{H}^+}$ ,

the electrochemical potential difference for protons (that is,  $\text{H}^+$  ions) between the two aqueous phases, by

$$\Delta p = \Delta\tilde{\mu}_{\text{H}^+}/F = \Delta\psi - (RT/F)\ln(10)\Delta\text{pH} \quad (1)$$

where  $R$ ,  $T$ , and  $F$  denote the gas constant, the absolute temperature, and the Faraday constant, respectively [at 30°C,  $(RT/F)\ln(10) \approx 59$  mV].

Most techniques used to measure  $\Delta\psi$  in mitochondria fall into two broad categories: (a) the introduction of non-physiological chemicals, which either render the mitochondrial membrane permeable to some ion [2] or are themselves both charged and membrane-permeant [3–5], so allowing calculation of  $\Delta\psi$  from the equilibrium measurement of transmembrane diffusion; or (b) a non-physiological manipulation of mitochondrial structure, increasing mitochondrial volume enough to allow direct measurement of  $\Delta\psi$  by impalement with a microelectrode [6–11]. Ion-distribution methods generally measure a  $\Delta\psi$  in line with the chemiosmotic theory; controversially, however, microelectrode-based methods have found a negligible  $\Delta\psi$ . This very long-standing paradox at the core of bioenergetics has attracted numerous interpretations but, in the view of many, remains unresolved. There is thus a case for re-

\* Tel.: +44-1223-333963; fax: +44-1223-333992; e-mail: ag24@gen.cam.ac.uk

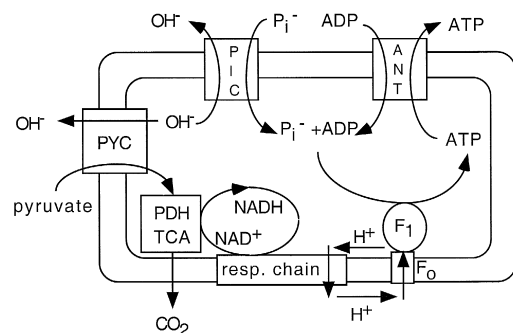


Fig. 1. A summary of relevant mitochondrial proteins and their substrates. ANT, adenine nucleotide carrier;  $F_1$ ,  $F_0$ , ATPase components; PDH, pyruvate dehydrogenase; PIC, phosphate carrier; PYC, pyruvate carrier; TCA, tricarboxylic acid (Krebs) cycle.

newed analysis of it, possibly incorporating new data from ostensibly unrelated fields; such an analysis, culminating in a new hypothesis to explain the above findings, is presented here.

## 2. Validity of the experimental techniques

The main challenge to ion-distribution measurements of  $\Delta\psi$  has been that their uptake may be electroneutral [10] and thus not driven by  $\Delta\psi$ . However, this challenge seems hard to sustain. One of the ions most often used to measure  $\Delta\psi$  is rhodamine 123 [3]; that  $\Delta\psi$  is indeed the driving force for its uptake has been shown in many ways [4]. For example, uptake is abolished by ionophores that dissipate  $\Delta\psi$ ; conversely, it is much enhanced by nigericin, which reduces  $\Delta\text{pH}$  by allowing  $\text{H}^+/\text{K}^+$  exchange and thereby allows the respiratory chain to increase  $\Delta\psi$  [12].

Conversely, all challenges to the microelectrode technique have also been robustly met. The electrode was shown to be really inside the mitochondrion, rather than encased in an invagination of its (unpunctured) membrane or in one of multiple vesicles, by double impalement [9]. The impaled mitochondrion retained ability to synthesise ATP, since a nearby myofibril contracted on introduction of ADP and phosphate, but not when OXPHOS inhibitors were also added [8]. The consistently observed membrane resistance of 2 M $\Omega$  was challenged as abnormally low [13], implying that the electrode did not seal well, only by a method that neglected flow of ions other than protons [7]; besides, even if the resistance was less than normal, it was evidently enough to sustain ATP synthesis. Finally, the mitochondrion's internal pH (hence,  $\Delta\text{pH}$ ) was normal [11].

This conflict of highly compelling data, relating to so fundamental a physiological process, surely merits energetic scrutiny until it is resolved. However, the recent tendency has been to polarise the theoretical situation, describing it [14] as a choice between Mitchell's "delocalised" scheme (Fig. 2a) and a "localised" proton circuit

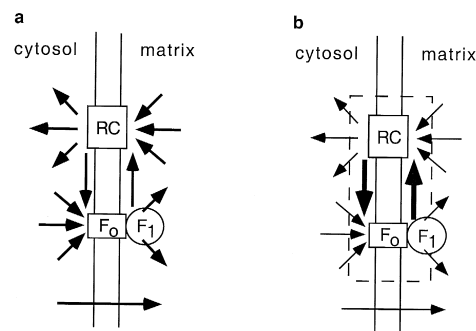


Fig. 2. Delocalised (a) and localised (b) proton circuits. The localised circuit incorporates a resistance to proton conduction (dotted line) which will cause slower stimulation of respiration by ionophores than by ADP; this is generally not seen (reviewed in Ref. [14]).  $F_0$ ,  $F_1$ , ATPase components; RC, respiratory chain.

(Fig. 2b) in which the proton pumps exchange protons by a direct interaction, from which they rarely escape — a scheme against which there is indeed good evidence, noted below. This is an oversimplification, in view of the long-standing availability [15–17] of models following neither paradigm.

## 3. "Electrodic" proton circuits

One such model [15] derives from the Grotthuss mechanism [18] of proton conduction in water [19], and concludes that conduction near the membrane surface is modulated by the organisation of the (dipole) water molecules there — similar to that at an electrode — which is caused by the fixed negative charges on the phospholipid head groups (Fig. 3a; Ref. [20]). Specifically, this organisation promotes conduction across the face of the membrane and

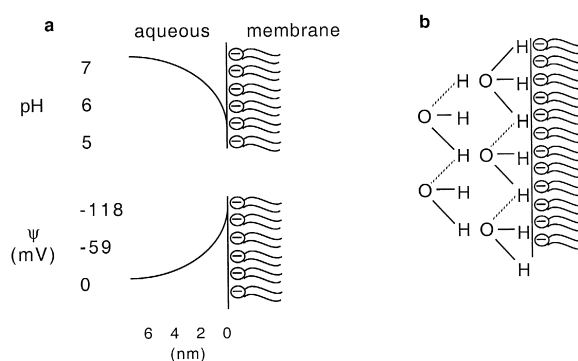


Fig. 3. Organisation of water impedes proton conduction perpendicular to the membrane. (a) The variation of potential ("surface potential") and pH near an anionic membrane. In the absence of proton pumping, these cancel out so that the whole aqueous phase is in electrochemical equilibrium [43]. The size of these effects is sensitive to membrane composition; the coordinates shown are typical of the outer face of the mitochondrial inner membrane. (b) The arrangement of water molecules near an anionic membrane, including a molecule of hydronium (top right): since they are dipolar, the surface potential orients them as shown. Note that hydronium is pyramidal [69] and thus also a dipole. The dotted lines indicate hydrogen bonds.

impedes conduction perpendicular to it (Fig. 3b). Thus, the proton circuit would be mostly localised to the vicinity of the membrane but delocalised within that domain (Fig. 4a); hereafter, this will be termed a *surface-delocalised* proton circuit. Such a circuit was proposed [15] to allow a PMF between the surface and the bulk (hereafter, denoted  $\delta p$ ; see Table 1), in contrast to the fully delocalised model where proton transfer between these domains is rapid and  $\delta p$  must thus be zero. This would allow a PMF across the proton pumps (hereafter, termed  $\partial p$ , composed of  $\partial \psi$  and  $\partial pH$ ) exceeding that between the bulk phases (Fig. 4b), so that ATP could after all be synthesised with a negligible  $\Delta p$ . The model is also consistent with the result most often cited as challenging the fully “localised” models mentioned above, namely that ionophores and ADP induce indistinguishable kinetics of stimulation of electron transport (reviewed in Ref. [14]), implying that any resistance to protons’ departure from the OXPHOS circuit (Fig. 2b) is negligible. [It should be stressed, however, that there has been much controversy (e.g., Refs. [21,22]) regarding both this result and its compatibility with the competing hypotheses.] Support for Kell’s model, in the form of evidence for the inhibition of conduction perpendicular to a charged membrane, is now extensive [23–26]. On the other hand, it cannot be ignored that others have found no significant inhibition of such conduction [27,28] or have presented alternative explanations for some of the early observations of it [29].

Mitchell himself acknowledged [30] that surface water would conduct as just described, but he identified [31] a major flaw in the idea that this could be relevant to chemiosmosis. The surface-to-bulk transfer of protons may be retarded by the organisation of water molecules, but it will not be abolished: indeed, studies of kinetics of ATPase activation and inactivation (e.g., Refs. [27,32]) demonstrate that it is substantial. Therefore, a mitochondrion at steady state (of both respiration and ATP synthesis) cannot maintain a PMF between surface and bulk, since a non-zero  $\delta p$  would be inexorably dissipated by proton transfer, whatever the relative rates of proton flow parallel vs. perpendicular to the membrane. This has led to

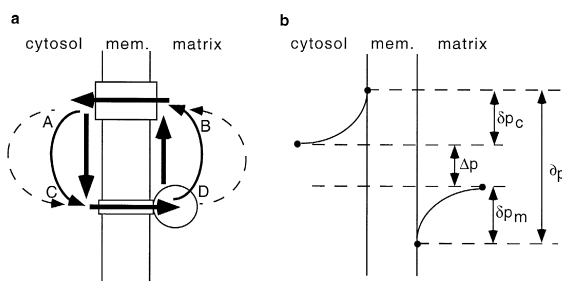


Fig. 4. (a) The original “electrodic” surface-delocalised proton circuit; (b) the proposed [15] consequences for PMF in various intervals. Note that the cytosol-to-matrix currents at C and D are required to equal the matrix-to-cytosol currents at A and B.

Table 1

Notation for PMFs and their components, between various sites

In each row, the PMF is the combination of the corresponding potential difference and pH difference, following Mitchell [1]; the symbols in the top row are standard usage.  $B_c$ ,  $B_m$ ,  $S_c$  and  $S_m$  are the regions shown in Fig. 5.  $\partial p$  is the PMF felt by the proton pumps;  $\Delta \psi$  is what is measured by either ion-distribution or microelectrode techniques.  $\delta \psi_c$  and  $\delta \psi_m$  are necessarily zero (after subtracting OXPHOS-independent surface effects) in all models discussed in this article; in the standard chemiosmotic theory  $\delta pH_c$  and  $\delta pH_m$  are also zero.

Interval	Potential difference	pH difference	PMF
Bulk-to-bulk, $B_c$ to $B_m$	$\Delta \psi$	$\Delta pH$	$\Delta p$
Surface-to-bulk	$\delta \psi$	$\delta pH$	$\delta p$
In cytosol, across $S_c$	$\delta \psi_c$	$\delta pH_c$	$\delta p_c$
In matrix, across $S_m$	$\delta \psi_m$	$\delta pH_m$	$\delta p_m$
Transmembrane, $S_c$ to $S_m$	$\partial \psi$	$\partial pH$	$\partial p$

the wholesale rejection [33] of the surface-delocalised proton circuit, even by its early proponents [34].

Such rejection is premature, however. The “proof” that the proton circuit is not surface-delocalised [31,33] starts from the assumption that the surface phases cover the entire membrane, including all transmembrane proteins, so making it topologically impossible for protons to move between the bulk phases without, at some point, coming into electrical contact with the surface water. It was inferred [33] that non-Mitchellian coupling could result only from some unidentified heterogeneity of the membrane’s lipid or protein components. The idea that well-known membrane components might suffice was not entertained.

In this connection, it is necessary to recall that the mitochondrial inner membrane is relatively permeable to small neutral molecules, especially  $H_2O$  [35]. Thus, transmembrane proton transport in one direction is equivalent to transport of hydroxide anions ( $OH^-$ ) in the other, since they are interconverted by diffusion of  $H_2O$ .

#### 4. $OH^-$ transport by respiring mitochondria

Several transmembrane metabolite carriers must operate in order to maintain mitochondrial OXPHOS at steady state. The two with fastest turnover (summed over a whole mitochondrion — the number of individual carriers per mitochondrion is not relevant here) are the adenine nucleotide and phosphate carriers, which must each cycle once for every ATP molecule synthesised within the mitochondrion (and not re-hydrolysed there). The phosphate carrier is relevant to the present discussion because it operates by antiport with hydroxide anions. (Traditionally, it has been considered impossible to distinguish between hydroxide antiport and proton symport, due to the membrane’s permeability to  $H_2O$  noted above, but the careful kinetic studies in the laboratory of Krämer [36,37] strongly indicate that the phosphate carrier uses hydroxide.)

Let us now compare the behaviours of a proton and a hydroxide ion when they are transported between the aqueous phases by a mitochondrial transmembrane carrier. In each case, there is a location at which the charge carrier loses its electrochemical contact with the surrounding water, and a location on the other side of the membrane at which it resumes such contact. How far are these sites from the plane of the membrane surface?

The detailed mechanisms of proton pumping are not fully established, but there is strong experimental support for Mitchell's "Q-cycle" hypothesis of electron transfer to Complex III coupled to proton translocation [38], in which protons cross the membrane bound to ubiquinone. Complex I may use a similar mechanism [39]. Ubiquinone is highly hydrophobic, so its exchange of protons with water (at either complex) should occur exactly at the lipid/water boundary line on the protein surface, even though the protein complexes with which it exchanges electrons protrude substantially from the membrane. (Complex IV does not interact with ubiquinone, but its mechanics — at least with regard to the precise location of the "ends" of its proton channel(s) — are still too unclear for speculation on this point to be productive.) Current models of Complex V [40] suggest that it also interacts with water at — or even below — the membrane surface.

### 5. Hypothesis: $\text{OH}^-$ transport is bulk-to-bulk

Hydroxide, by contrast, is transported mainly by a carrier protein (the phosphate carrier) which comprises a zig-zag arrangement of transmembrane helices separated by extensive hydrophilic stretches [41]. Thus, though its tertiary structure is still unknown, the carrier must extend at least 1 nm or so from the membrane on either side. This allows the possibility that it interacts with water further from the plane of the membrane surface than do the ubiquinone-dependent proton pumps. It is here proposed that, indeed,  $\text{OH}^-$  capture and release occur on the order of 1 nm from the plane of the membrane surface, and are thus separated from it by a few layers of water molecules. As a  $\text{OH}^-$  ion moves between these points it passes through the plane of the surface water, but it is proposed to be insulated from that water by the carrier protein. This arrangement may be necessary for rapid turnover of the carrier, since both  $\text{OH}^-$  and  $\text{P}_i^-$  concentrations — hence, rate of binding to the carrier — are lowered at the surface by repulsion of the phospholipid head groups surrounding the carrier (Fig. 3a; Ref. [20]).

With this in mind, we may regard the cytosolic and matrix phases as each divided into a bulk phase,  $B_c$  and  $B_m$ , respectively, and a surface phase,  $S_c$  and  $S_m$ . This follows the original electrodic model (Fig. 4; Ref. [15]), except that the boundaries between surface and bulk are here defined as the perpendicular distances from the membrane surface at which  $\text{OH}^-$  capture and release occur.

The implications of this model for the steady-state distribution of hydronium near the mitochondrial inner membrane are considerable (Fig. 5). There is a continuous flow of  $\text{OH}^-$  through the phosphate carrier from  $B_m$  to  $B_c$ , which is equivalent to proton flow from  $B_c$  to  $B_m$  so must — at steady state — be matched by a flow of protons from  $B_m$  to  $B_c$ . The transmembrane proton pumps move these protons only part of this distance, from  $S_m$  to  $S_c$ ; the regions  $S_c$  and  $S_m$  must be crossed by a transfer of protons between water molecules free in solution. The water in these regions is organised, as described above, so there is some resistance to this transfer.

This implies, on the cytosolic side, that there must indeed be a non-zero PMF, driving that proton current against that resistance, across the aqueous phase  $S_c$ : that is, a non-zero  $\delta p_c$ . There will be residual perpendicular proton transfer (both by the Grotthuss mechanism and by simple diffusion of hydronium, which accounts for about 4% of proton movement in bulk water [19]), but this can never dissipate  $\delta p_c$  as long as protons continue to arrive in  $S_c$  via the respiratory chain. Ions other than hydronium will also diffuse freely between surface and bulk, however, so at constant respiration rate  $\delta p_c$  will have no electrical component and will be composed wholly of osmotic pressure ( $\delta p_H$ ). [This ignores the surface potential, which in the absence of respiration is exactly cancelled out by a pH gradient [42,43] (Fig. 3a) so does not alter  $\delta p_c$ , but whose profile is likely to be somewhat altered by the  $\delta p_c$  induced by the proton flows just described. The nature of that alteration may be complex, but should not qualitatively

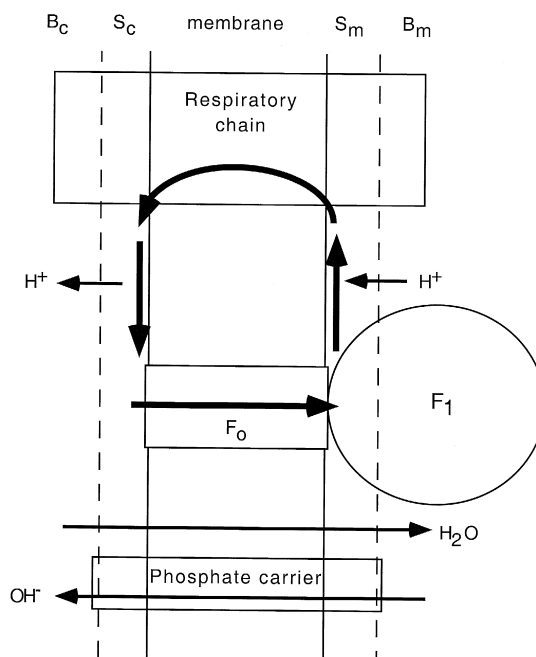


Fig. 5. The interaction of the proton circuit with hydroxide transport. The consequences for PMFs are as in Fig. 4b. Phosphate transport is omitted for clarity, since at steady state its associated charge transfer is exactly neutralised by ATP/ADP transport so plays no role.

alter the situation.] A symmetrical situation obtains on the matrix side.

Thus,  $\partial\text{pH}$  exceeds  $\Delta\text{pH}$  by the sum of  $\delta\text{pH}_c$  and  $\delta\text{pH}_m$ . The  $\Delta\psi$  ( $=\partial\psi$ ) needed for ATP synthesis is thus correspondingly reduced. It is proposed that, in the giant mitochondria of Tedeschi's group, the sum of  $\delta p_c$  and  $\delta p_m$  was on the order of 200 mV, so allowing ATP synthesis with near-zero  $\Delta p$ .

## 6. Buffering of $\Delta p$ against loss of $\delta p$

The organisation of surface water, on which  $\delta p$  absolutely depends, is disrupted by chaotropes such as thiocyanate, or indeed by any substance that reduces the surface potential [15,44] — in the latter case, this arises because the surface water will orient randomly in the absence of the electric field. They thus cause reduction of  $\partial p$  to  $\Delta p$ . Loss of  $\delta p$  would also result from loss of  $\text{OH}^-$  transport (due, for example, to lack of cytosolic  $\text{P}_i^-$ ). It may thus be a frequent, albeit transient, occurrence in vivo, with an impact on ATP supply. Thus, a homeostatic mechanism may well exist to maintain  $\partial p$  at such times; a natural way to do so would be to create a  $\Delta\psi$ . This may be achieved by regulating the nucleotide-sensitive  $\text{K}^+$  channel [45], which is already implicated in control of  $\Delta\psi$  [46].

Such a creation of a  $\Delta\psi$  in response to collapse of  $\delta\text{pH}$  would readily explain the disparate measurements of  $\Delta\psi$  by ion distribution and by microelectrodes, since the ions used are of just the type to disorganise surface water as described. It is postulated that  $\delta p$  would be dissipated even at the sub-micromolar concentrations of probe that are often used. Such concentrations are comparable to those required for an ionophore to induce significant uncoupling [47]. It is proposed that ion-distribution measurements do indeed reflect a real bulk-to-bulk  $\Delta\psi$ , but that this is a  $\Delta\psi$  which was absent (or, at least, less) until the ion was introduced. Conversely,  $\delta p_c$  and/or  $\delta p_m$  may be greater in giant mitochondria than in normal ones. We may conclude that neither assay can reliably report the in vivo  $\Delta\psi$ .

This does not, however, imply that ion-distribution measurements of  $\Delta\psi$  are unreliable indicators of  $\Delta p$ . The homeostatic mechanism postulated above would be expected to maintain the rate of ATP synthesis — and, hence,  $\partial p$  — which obtained before the ion was introduced. The only effect of the ion, therefore, is to raise  $\partial\psi$  (and thus  $\Delta\psi$ ) by an amount equivalent to the lost  $\partial p$ .

## 7. Consistency with other data

If we neglect the effect of transmembrane proton leak [48] and of other proton or hydroxide transport (such as by the pyruvate carrier), and adopt the consensus stoichiometry

of Complex V of four protons per ATP, we may deduce that only 4/5 of the protons released by the respiratory chain enter Complex V and fully 1/5 escape into the bulk cytosol, re-entering as “water minus hydroxide” as depicted in Fig. 5. This means that the present model is consistent with two important findings [27,28,49] which were originally brought to bear on opposite sides of the “localised–delocalised” debate — that is, presented as inconsistent with localised [27,28] and delocalised [49] chemiosmosis. The surface-to-bulk conductance is enough to allow the observed rapid response of ATP synthesis to an artificially imposed  $\Delta\psi$  [27] and rapid exchange of protons between surface and bulk [28]. But this conductance also predicts that protons leaving a particular respiratory chain enzyme complex will only enter very nearby ATPases, since they would escape to the bulk before travelling further; this appears as the “mosaic” coupling [34] so strongly indicated by double-inhibitor titration data [49]. (The latter argument only works if most protons entering a given Complex V become irreversibly committed to pass through it, rather than to escape back to the cytoplasmic side, since the rate of turnover of Complex V [50] is too slow to produce mosaic coupling otherwise — protons trapped at the surface would on average have time to visit every ATPase in the mitochondrion in the time that a given ATPase makes one ATP. But irreversible entry seems compatible with current models of the ATPase proton channel [40].) No coupling mechanism has previously been proposed that reconciles these two important bodies of data while also accounting for the indistinguishable kinetics of stimulation of electron transport by protonophores and ADP (mentioned earlier).

## 8. Tests of this hypothesis

### 8.1. Measurement of $\Delta\psi$ in giant mitochondria with fluorescent ions

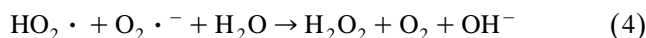
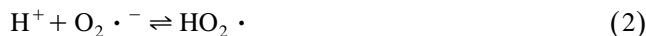
Tedeschi's group also measured the  $\Delta\psi$  of giant mitochondria using a variety of fluorescent dyes, and reported values inadequate for ATP synthesis, just as they had when measuring  $\Delta\psi$  by microelectrodes [51–53]. However, these results were much more equivocal than the microelectrode findings, in that some dyes did distribute so as to imply a  $\Delta\psi$  of up to 60 mV induced by respiration. The authors presented arguments suggesting that the other dyes used, which showed a much smaller induced  $\Delta\psi$ , were more reliable; but, crucially, these experiments were not followed up in the same comprehensive manner as the microelectrode work (in particular, demonstration of ATP synthesis). It is therefore possible that the preparation of giant mitochondria in these experiments [51–53] rendered them less able to compensate for a collapse of  $\delta p$  by generating  $\Delta\psi$  as proposed above. The hypothesis presented here predicts that giant mitochondria will be unable

to synthesise ATP in the presence of dyes which measure a large  $\Delta\psi$  in normal mitochondria (i.e., cause a collapse of  $\delta p$  in this model) but do not measure a significant one in giant mitochondria. The much more reliable dyes now in use for this purpose should allow this prediction to be tested more rigorously than would have been possible at that time.

## 8.2. Impact of OXPHOS on surface pH

The main determinant of  $\delta p H_c$  and  $\delta p H_m$  is the rate of phosphate import, since  $\delta p$  determines the rate of transfer of protons across  $S_c$  and  $S_m$ , which must match that of  $OH^-$  movement. This in turn is set, at steady state, by the respiration rate. Thus, since the absolute pH in  $B_c$  is hugely buffered and that in  $B_m$  hardly less so, the absolute pH within  $S_c$  and  $S_m$  will vary with respiration rate. Specifically, a rise in respiration lowers the pH in  $S_c$  and raises it in  $S_m$ .

This opens the possibility of estimating  $\delta pH$  of respiring mitochondria by chemical means. Indeed, one such result is already available. Work in the laboratory of McCord [54] has shown that respiration of isolated mitochondria markedly accelerates the nonenzymatic dismutation of superoxide radical ( $O_2 \cdot^-$ ) generated extramitochondrially by xanthine oxidase. This result is relevant because the dismutation reaction is pH-dependent, requiring the protonation of  $O_2 \cdot^-$  to perhydroxyl radical,  $HO_2 \cdot$  – the relative rates of reactions (3), (4) and (5) below are about  $10^6:10^8:1$  [55].



Since the bulk pH is unaffected by respiration, the increased acidity must be at the mitochondrial surface; this implies a respiration-dependent  $\delta p H_c$ . [The pH in  $S_m$ , conversely, is predicted to *rise* with respiration, but we can ignore this: the  $pK$  in reaction (2) is 4.7 [55], well below the pH in  $S_c$  or  $S_m$  in the absence of respiration [42], so the rise in pH in  $S_m$  will have less effect on the overall levels of  $HO_2 \cdot$  than does the fall in pH in  $S_c$ , even if  $O_2 \cdot^-$  or  $HO_2 \cdot$  diffuse rapidly across the membrane.] Two alternative interpretations were eliminated: (i) the effect was truly respiration-dependent, because it was removed by either the ionophore FCCP or the respiratory chain inhibitor antimycin; and (ii) it could not be due to respiration-dependent production of  $O_2 \cdot^-$ , because  $O_2 \cdot^-$  concentration was raised (by xanthine oxidase) far above what respiration-dependent processes could generate. This observation, together with the identification of  $HO_2 \cdot$  as

the main initiator of lipid peroxidation in the inner membrane [56], also supports a recently proposed mechanism [57] to explain several hitherto mysterious aspects of mitochondrial population dynamics. Furthermore, there is now strong histochemical evidence [58] that non-enzymatic dismutation of superoxide occurs in the mitochondrial intermembrane space *in vivo*, which requires a pH well below that in the bulk cytoplasm (see above).

## 9. Other energy-transducing membranes

A circumstantial point in favour of the model presented here is that thylakoid membranes seem to rely wholly on  $\Delta pH$  to drive ATP synthesis [59]. The topology of chloroplasts is such that no hydroxide (or phosphate) transport across the thylakoid membrane accompanies ATP synthesis, so no  $\delta pH$  can result from the model presented here: that is, chloroplast coupling is purely Mitchellian. Evidence against this was obtained by Hauska et al. [60], who reconstituted the photosystem I reaction centre and coupling-factor complex in liposomes and saw only 2% of the turnover number observed in intact chloroplasts; but this may have been due to failure to sustain adequate  $\Delta pH$ , since this was measured by 9-aminoacridine, which overestimates  $\Delta pH$  by at least a unit [61].

Bacteria, however, operate with a  $\Delta pH$  inadequate for ATP synthesis, so in the standard model [1,62] they require a  $\Delta\psi$ . Moreover, their topology does not require transmembrane phosphate transport for ATP synthesis. However, the import of various nutrients (such as lactose, whose carrier is commonly considered as a paradigm of a  $H^+$ /anion symporter [63]) may instead be coupled to hydroxide export, since the lactose carrier is similar in secondary structure to the homodimeric phosphate carrier; in that case, a  $\delta pH$  could exist as proposed for mitochondria. A report [64] that bacterial  $\Delta\psi$  measured with microelectrodes was OXPHOS-competent can be reconciled with the model presented here by the possible lack of substrate transport in highly stressed cells at the limit of the size inducible by mecillinam. Measurements using carotenoid electrochromism, notably, give values of  $\Delta\psi$  in illuminated or NADH-oxidising chromatophores much higher or lower, respectively, than that suggested by ion uptake, suggesting that they measure a different aspect of the energised state [65]; if carotenoids truly respond to  $\partial\psi$ , then this phenomenon is at odds with delocalised chemiosmosis [66] but could be accommodated within the model presented here. Finally, it should be noted that the  $\delta pH$  at the two sides of the membrane contribute independently to the effect proposed here; thus, for example, the 20 Å protrusion of the *Rhodobacter* reaction centre's H subunit (and, presumably, proton channel) into the cytoplasm must be considered in the context that the L and M subunits protrude hardly at all into the periplasm [67].

## 10. Control of cation uptake

The constitutive maintenance of a large  $\Delta\psi$  is, teleologically, a significant liability for the mitochondrion, since any permeant cation which enters the cytosol will accumulate in the matrix unless actively exported. Such export mechanisms are necessarily very ion-specific, so ions will inevitably exist for which there is none; these may present a major hazard. The model presented here — which invokes no unidentified mitochondrial substructure, and whose consistency with experimental data appears greatly to exceed that of the standard chemiosmotic theory [1,62] — suggests that by coupling metabolite transport to hydroxide transport in the appropriate direction to generate  $\partial p$  without  $\Delta p$ , nature has removed that hazard without sophisticated augmentation of the core OXPHOS machinery.

Finally, it is necessary to consider the well-known *desirable* aspects of cation accumulation, particularly of  $\text{Ca}^{2+}$ . The standard view — that  $\text{Ca}^{2+}$  is constantly propelled into the matrix by  $\Delta\psi$  and re-ejected at the expense of proton import — is challenged by the model presented here. An alternative model presents itself, however. If the cation antiporters are (like the proton pumps) transporters of ions between  $S_c$  and  $S_m$ , then  $\partial p$ , rather than  $\Delta p$ , is the force available to drive  $\text{Ca}^{2+}$  ejection: thus, that half of the machinery needs no revision.  $\text{Ca}^{2+}$  uptake, conversely, can be brought about on demand by up-regulation of  $\Delta\psi$ , as proposed above for buffering against collapse of  $\delta p$ ; calcium thereby imported is then rendered osmotically inactive by phosphate, preventing it from diffusing out again when  $\Delta\psi$  is reduced. Thus, suitable regulation of ion transport can certainly achieve  $\text{Ca}^{2+}$  homeostasis in the context of the proton circuit presented here. Moreover, candidate loci of such regulation are readily to hand. One is the nucleotide-sensitive  $\text{K}^+$  channel mentioned earlier [45]; another is suggested by the evidence of environmentally induced changes in stoichiometry of the heart  $\text{Na}^+/\text{Ca}^{2+}$  antiporter [68].

## Acknowledgements

I am indebted to Douglas Kell for many illuminating exchanges and to Adelaide Carpenter, Martin Brand and anonymous reviewers for valuable comments on the manuscript.

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