Chemiosmotic misunderstandings

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Abstract

Recent publications have questioned the appropriateness of the chemiosmotic theory, a key tenet of modern bioenergetics originally described by Mitchell and since widely improved upon and applied. In one of them, application of Gauss' law to a model charge distribution in mitochondria was argued to refute the possibility of ATP generation through H^+ movement in the absence of a counterion, whereas a different author advocated, for other reasons, the impossibility of chemiosmosis and proposed that a novel energy-generation scheme (referred to as "murburn") relying on superoxide-catalyzed (or superoxide-promoted) ADP phosphorylation would operate instead. In this letter, those proposals are critically examined and found to be inconsistent with established experimental data and new theoretical calculations.

Text

The modern chemiosmotic theory[1–3], supported by a wealth of experimental data collected over more than 60 years, posits that the energy released when electrons are transferred from a low-potendial donor to a high-potential acceptor through the mitochondrial electron-transport chain is used to expel protons (H^+) from the mitochondrial matrix and into the intermembrane space. The ensuing cross-membrane-difference in pH (ΔpH) adds to the mitochondrial membrane potential (ΔE) yielding an energetic proton-motive force (Δp) given by the equation

$$\Delta p = \Delta E - \frac{RT \ln(10) \Delta pH}{F} \quad (1)$$

When the protons return to the matrix through the membrane-bound ATP synthase this energy is released to drive the phosphorylation of ADP, thereby yielding ATP[4,5]. This mechanism is not limited to mitochondria, and is operative also in chloroplasts [6] and in prokaryotic organisms[7], both aerobic and anaerobic. In spite of its widespread adoption and acclaim, significant opposition to the theory was voiced, especially in its early years[8,9] and a few of the initial details proposed by Mitchell (such as the full attribution of the electrostatic component to the difference in proton concentrations across the membrane, or the specific H^+/ATP ratio proposed) have since been discarded or modified.

In a recent contribution in these pages[10], Nath ingeniously used Gauss' flux theorem to compute the electric potential in a model mitochondrion and obtained an extremely high positive value (86×10^5 V). From this, Nath concluded that the current formulation of the chemiosmotic theory is flawed and that a different mechanism for the energy coupling in oxidative phosphorylation is operative. Those calculations, however, suffer from an important flaw since they assume that the only charges present are the positive charges in the protons (located *inside* the mitochondrion) and that no charges of opposite sign are left behind in the compartment that the protons originated from (Figure 1A). A correct representation of the chemiosmotic model, in contrast, must place the protons outside the mitochondrion and, most importantly, take into account the negative charges remaining *inside* the mitochondrion to comply with the overall conservation of charge in the system (Figure 1B).

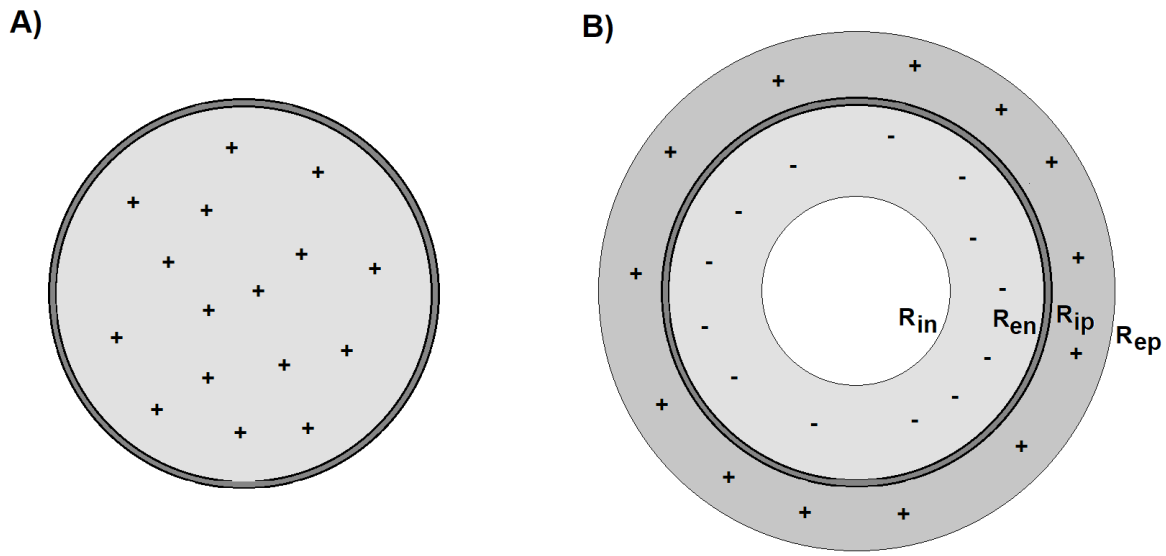

Figure 1: Comparison of the Nath model (A) and the correct model (B) of charge distribution on a model spherical mitochondrion. Distances to the center are highlighted as Ren (external edge of the negative shell), Rin (internal edge of the negative shell), Rip (internal edge of the positive shell), and Rep (external edge of the positive shell)

Application of Gauss' flux theorem to this improved model now yields the following equation for the electric field at an arbitrary point at distance *r* from the center of the system:

$$E = \frac{q_{positive}}{4\pi\varepsilon r^2} + \frac{q_{negative}}{4\pi\varepsilon r^2} \tag{2}$$

, where $q_{positive}$ and $q_{negative}$ are the positive and negative charges enclosed in the spherical surface with radius *r* centered at the geometrical center of the system. This equation shows that at distances larger than R_{ep} or shorter than R_{in} the field is exactly zero, and at intermediate distances the field can be decomposed as follows

$$E = \begin{cases} q_{totalpositive}\dfrac{\left(r^3 - R_{ip}^3\right)}{4\pi\varepsilon_{solution} r^2\left(R_{ep}^3 - R_{ip}^3\right)} + \dfrac{q_{totalnegative}}{4\pi\varepsilon_{solution} r^2} & \text{for } R_{ip} \leqslant r < R_{ep} \\ \dfrac{q_{totalnegative}}{4\pi\varepsilon_{membrane} r^2} & \text{for } R_{en} < r < R_{ip} \\ q_{totalnegative}\dfrac{\left(r^3 - R_{in}^3\right)}{4\pi\varepsilon_{solution} r^2\left(R_{en}^3 - R_{in}^3\right)} & \text{for } R_{in} \leqslant r < R_{en} \end{cases} \tag{3}$$

Since the potential at a distance *r* from the center is given by

$$\varphi_r = -\int_{\infty}^{r} E dr \tag{4}$$

, it follows that the potential at a given distance from the center is given by

$$V=\begin{cases} 0 & \text{for } r>R_{ip} \\ \dfrac{\dfrac{q_{totalpositive}R_{ip}^3}{\left(R_{ep}^3-R_{ip}^3\right)}-q_{totalnegative}}{4\pi\varepsilon_{solution}}\left(\dfrac{1}{R_{ep}}-\dfrac{1}{r}\right)-\dfrac{1}{2}\dfrac{q_{totalpositive}\left(r^2-R_{ep}{}^2\right)}{4\pi\varepsilon_{solution}\left(R_{ep}^3-R_{ip}^3\right)} & \text{for } R_{ip}\leqslant r<R_{ep} \\ V_{Rip}+\dfrac{q_{totalnegative}}{4\pi\varepsilon_{membrane}}\left(\dfrac{1}{r}-\dfrac{1}{R_{ip}}\right) & \text{for } R_{en}<r<R_{ip} \\ V_{Ren}+\dfrac{q_{totalnegative}\dfrac{R_{\text{in}}^3}{\left(R_{en}^3-R_{\text{in}}^3\right)}}{4\pi\varepsilon_{solution}}\left(\dfrac{1}{R_{en}}-\dfrac{1}{r}\right)-\dfrac{1}{2}\dfrac{q_{totalnegative}\left(r^2-R_{en}{}^2\right)}{4\pi\varepsilon_{solution}\left(R_{en}^3-R_{\text{in}}^3\right)} & \text{for } R_{\text{in}}\leqslant r<R_{en} \\ V_{Rin} & \text{for } r<R_{\text{in}} \end{cases} \quad (5)$$

From the above equations, it is apparent that the magnitude of the decrease in electric potential in each charged shell is completely independent of the thickness of the other spherical shell, since no term depending on the position of the boundaries of the internal shell (R_{en} or R_{in}) is present in the equation for the potential in the region between R_{ep} and R_{ip} (the external shell), and the effect of the external shell on the potential inside the mitochondrion is a constant throughout the radius of the mitochondrion. Moreover, the electric potentail can only decrease as one approaches the geometric center of the system, i.e., positively charged species present in this setup will tend to move to the inside of the mitochondrion, as postulated by the chemiosmotic theory and in contrast to the model in [10].

A hypothetical spherical model matching the measured volume of a mitochondrion (0.29 μm^3 [11]) must possess a radius of 411 nm. The resulting potential profiles for this model mitochondrion supplemented with a 5 nm-thick membrane and charged shells of varying thicknesses are depicted in Figure 2. The computed capacitance varies from 0.4 $\mu F/cm^2$ (for charges present in infinitely thin spherical shells adjacent to the membrane) to 0.14 $\mu F/cm^2$ (assuming that the negative charge is homogeneously diffused thoughout the mitochondrion and that the protons diffuse outwards into a shell 256 nm thick), within an order of magnitude of the experimental values for mitochondrial (0.5 $\mu F/cm^2$ [12]) or neuron membranes (0.9 $\mu F/cm^2$[13]). Since the equations above show that the magnitude of the electric potential at each point is strictly proportional to the amount of charge transferred from the mitochondrion to the cytoplasm, the total contribution of the charge separation induced by proton ejection to the membrane potential can be directly computed from the net number of charges that have been transferred from the mitochondrial matrix to the intermembrane space.

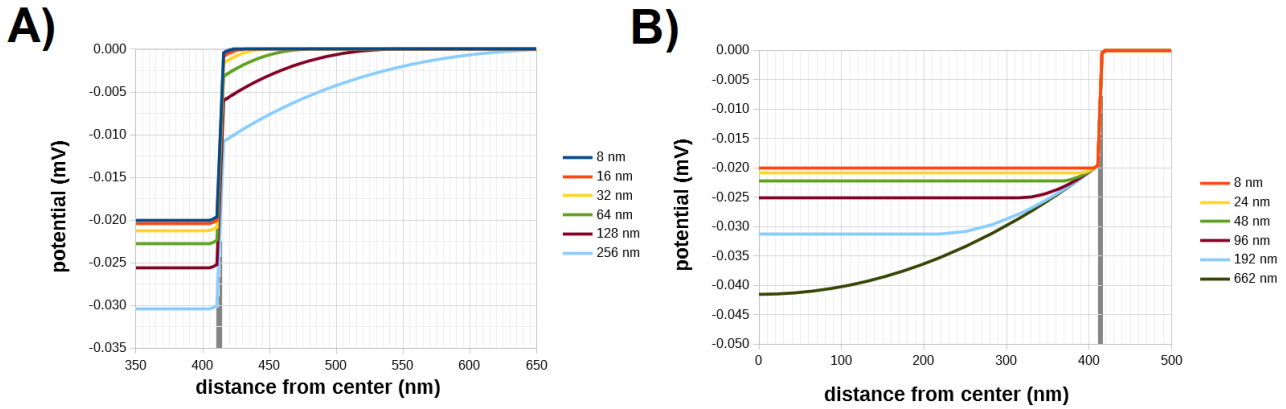

Figure 2: Influence of the outer (A) and inner (B) shell size on the electric potential (at different distances from the model mitochondial center) produced by the transfer of a single proton from the matrix to the intermembrane space. The position of the mitochondrial membrane is shown as grey bars. Effects of the outer and inner shells are independent and additive. In A), an inner shell with negative charge with 8 nm thickness is present, whereas in B) the cations are placed in an outer shell 8 nm thick. Folowing common practice, $\varepsilon_{membrane}$ was set to 2.2 and $\varepsilon_{solution}$ to 80

Although the number of protons available at initial pH between 7.0 and 7.5 is very small (between 17 and 6, respectively), sizeable charge separations can nonetheless be attained because every ejection of a proton from the matrix dislocates the auto-protolysis equilibrium of water towards the formation of new H^+ and HO^-. In the absence of an acid/base buffer, the number of new protons (and hydroxides) produced through auto-protolysis can be readily computed from the expression for the auto-protolysis equilibrium:

$$10^{-14} = [H^+] \cdot [HO^-] \quad (6)$$

$$10^{-14} = \frac{n_{H^+}}{v_{mitochondrion}} \frac{n_{HO^-}}{v_{mitochondrion}} \quad (7)$$

Replacing $v_{mitochondrion}$ with the experimentally measured volume (0.29 μm^3), we can calculate the number of new protons and hydroxides ($n_{new\ H+}$ and $n_{new\ OH-}$) generated through water auto-ionization as a function of the number of protons ejected from the matrix:

$$304.98 = n_{H^+} \cdot n_{HO^-} \quad (8)$$

$$304.98 = \left(n_{initial\ H^+} - n_{transferred\ H^+} + n_{new\ H^+}\right) \cdot \left(n_{initial\ HO^-} + n_{new\ HO^-}\right) \quad (9)$$

$$304.98 = \left(n_{initial\ H^+} - n_{transferred\ H^+} + n_{new\ H^+}\right) \cdot \left(n_{initial\ HO^-} + n_{new\ H^+}\right) \quad (10)$$

and solving the resulting quadratic equation returns

$$n_{new\ H^+} = \frac{n_{transferred\ H^+} - n_{initial\ HO^-} - n_{initial\ H^+} + \sqrt{\left(n_{initial\ HO^-} + n_{initial\ H^+} - n_{transferred\ H^+}\right)^2 + 4\, n_{initial\ HO^-} n_{trar}}}{2} \quad (11)$$

This quadratic formula also shows that $n_{new\ H+}$ is always strictly inferior to the number of protons trasnferred to the inter-membrane space, and therefore the net effect of this process is, as expected, the progressive alkalinization of the mitochondrial matrix, depicted in Figure 3 for various combinations of initial mitochondrial pH and numbers of ejected protons. The experimentally

measured mitochondrial pH (7.8) and difference of pH between cytoplasm and mitochondria (0.7-0.8 pH units) [14] can therefore be obtained with the **net** ejection of 100 protons from the matrix (in a hypothetical unbuffered mitochondrial model). Combining this result with the previously computed potential arising from the ejection of a single proton (Figure 2) we obtain, for the value of the electric potential due to the proton gradient, an interval between -1.9 mV (for charges present in infinitely thin spherical shells adjacent to the membrane) and -5.2 mV (assuming that the negative charge is homogeneously diffused thoughout the mitochondrion and that the protons diffuse outwards into a shell 256 nm thick).

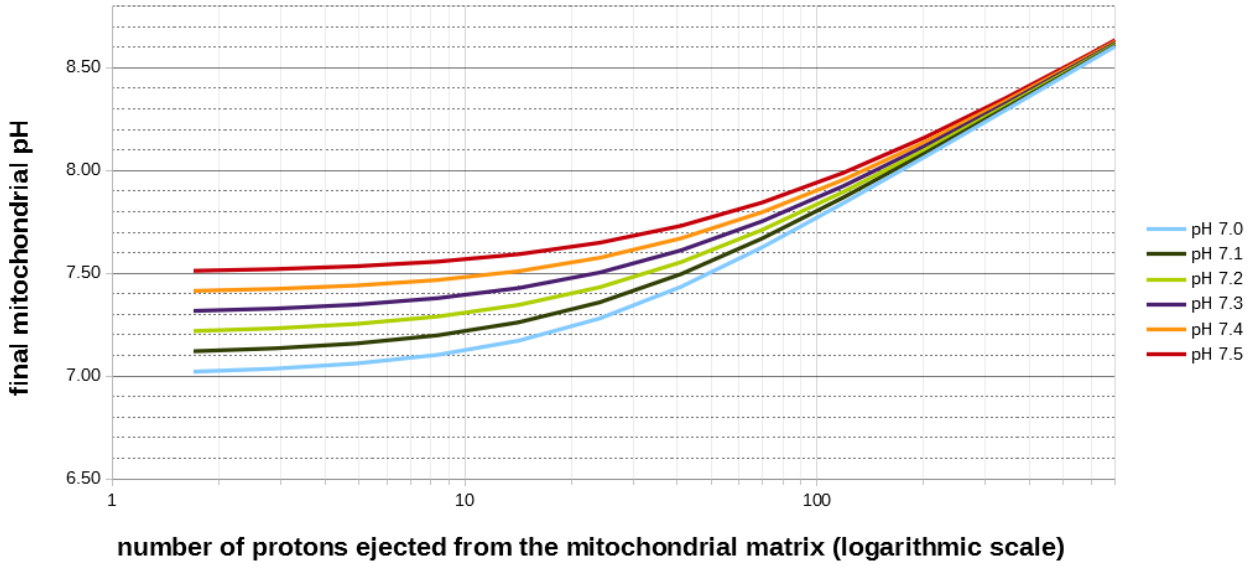

Figure 3:Changes of mitochondrial pH upon ejection of protons, for different values of initial pH, in the absence of internal pH buffers.

In the buffered environment of the mitochondria, however, the number of protons that must be ejected to obtain such a pH difference naturally depends on the concentration of the buffering species and on its pKa. Numerical analysis of the influence of the expulsion of increasing numbers of protons on the pH (Figure 4A) shows that this number is almost perfectly proportional to the concentration of the buffering species and can be as large as 76000 H^+/mM buffer in our model mitochondrion (if the buffer pKa lies in the middle of the interval, thus affording maximum buffering). If the buffer pKa lies farther from the center of the interval (e.g. taurine[15], with a pKa of 8.6), the number of protons required becomes lower (Figure 4B), but in any case the presence of buffer dramatically increases the number of protons that must be expelled to obtain a measureable proton difference, compared to the unbuffered example in Figure 3. Combining these numbers with the membrane capacitance computed earlier clearly shows that, absent other factors, the proton expulsion needed to generate a 0.8 pH difference across the membrane in the presence of as little as 10 mM taurine buffer in the mitochondrial matrix would entail the generation of an electric potential in excess of 1.9 V, more than enough to collapse the membrane (assuming that mitochondrial membranes, in spite of their higher protein content, behave similarly to cell membranes and breakdown at 4×10^6 V/cm[16]). While this might be taken to mean that the generation of a pH gradient through proton pumping is impossible, that will not be the case if mechanisms to dissipate the charge differential (while not affecting the pH difference) exist, either through the exit of anions from the matrix or through the uptake of cations. Indeed, extensive simulations [17] have established that, as the proton pumping proceeds, potassium entry in the mitochondria through both a K^+-uniport and a K^+/H^+ antiport occurs and the relative rates of these

two processes control the dissipation of the charge differential between both sides of the mitochondrial membrane and are mostly responsible for the bulk of the electric potential difference. It thus appears that the K^+-uniport system (which has mostly been thought to prevent mitochondrial matrix contraction[18]) may have a more important role in mitochondrial metabolism than previously envisioned. Other experimental support for the ultimately small contribution of ΔpH to ΔE comes from the observations that ΔpH can change without barely affecting ΔE [19] and vice-versa[14] and that other factors, not yet completely understood but which include complex II-driven electron flow [20], the charge separation provided by the exchange of mitochondrial ATP^{4-} with cytoplasmic ADP^{3-} [21], and the activity of accessory proteins to complex I [22] are also involved.

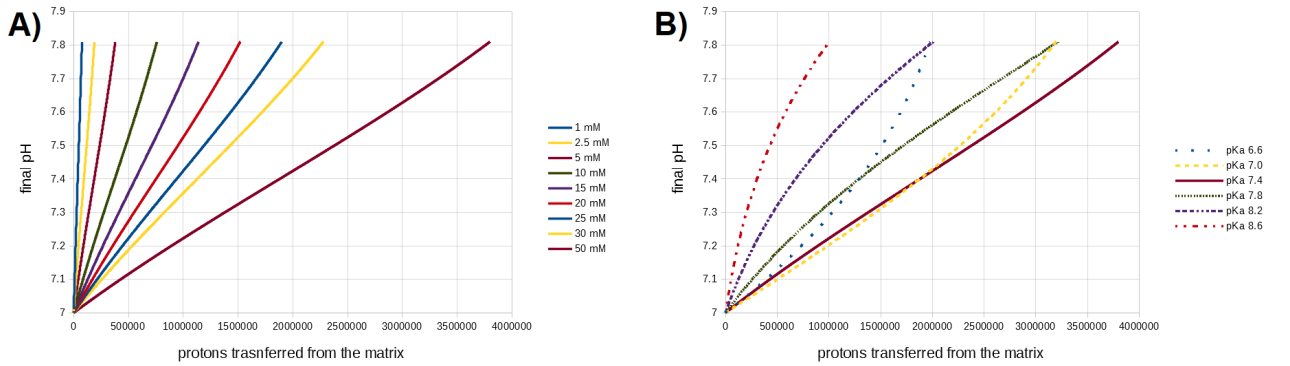

Figure 4: A) pH changes upon proton ejection from a model mitochondrion in the presence of variable concentrations of a buffer with pKa=7.4; B) pH changes upon proton ejection from a model mitochondrion in the presence of 50 mM of buffers with different pKa

The calculations above show that one of the reasons adduced by Nath to discard the chemiosmotic hypothesis as "catastrophic flaws and inconsistencies in chemiosmotic dogma" is actually not relevant. Other arguments provided by this author also lack force. For example, in an earlier work (Nath, 2017) this author argued that the unaccompanied movement of H^+ across the membrane represented a violation of the principle of electric neutrality. It is, however, not clear why that would be so: the return of the protons to the matrix is actually a process that tends to dissipate the charge separation and therefore moves the system towards electroneutrality. Electroneutrality itself is, moreover, not an absolute requirement in all circumstances, as attested by the existence of capacitators or by the generation of electric potentials across membranes through Donnan effects. A closer reading of the author's arguments suggests that their reasoning is based on the experimental observations of ATP synthesis in isolated liposomes bearing ATP-synthase in the absence of a proton-motive force or electron-trasport chains. Contrary to Nath's representations, however, such experiments are routinely performed after energizing the liposomes with baths of different pH and/or K^+/valinomycin concentrations[24,25], and are therefore readily explained and fully compatible with chemiosmotic theory. Another argument [26] notes Mitchell's unrigorous derivation[27] of the influence of electric potential on the equilibrium constant of ATP synthesis powered by an inter-membrane pH difference and claims that correcting this error implies that the potential difference must be zero at all times. A rigorous derivation, however, shows that the original result is valid. The synthesis of ATP in the inside of the membrane

$$ADP_{\text{in}} + Pi_{\text{in}} + 2\,H^+_{\text{in}} \rightarrow ATP_{\text{in}} + H_2O \qquad (12)$$

is coupled by ATP synthase with the transfer of *n* protons from the "out" side to the "in"side, yielding the following net reaction:

$$ADP_{\text{in}} + Pi_{\text{in}} + n\,H^+_{out} \rightarrow ATP_{\text{in}} + H_2O + (n-2)\,H^+_{\text{in}} \qquad (13)$$

For each species in the "in" (or "out") side, the chemical potential (μ) is given by

$$\mu_X = \mu_X^0 + z_X F E_{\text{in(or out)}} + RT \ln([X]) \tag{14}$$

, where z_X is the charge of species X, F is the Faraday constant and all other symbols have their usual meanings. The reaction energy would then be

$$\begin{aligned} \Delta\mu_{reaction} &= \mu_{ATP}^0 - \mu_{ADP}^0 - \mu_{Pi}^0 + (n-2)\mu_{H^+}^0 - n\mu_{H^+}^0 \\ &+ F\left(z_{ATP}E_{\text{in}} + (n-2)z_{H^+}E_{\text{in}} - z_{ADP}E_{\text{in}} - z_{Pi}E_{\text{in}} - n z_{H^+}E_{\text{out}}\right) \\ &+ RT \ln\left(\frac{[ATP]\left[H_{\text{in}}^+\right]^{(n-2)}}{[ADP][Pi]\left[H_{out}^+\right]^n}\right) \end{aligned} \tag{15}$$

, which simplifies to

$$\Delta\mu_{reaction} = \Delta\mu_{reaction}^0 + n F z_{H^+}\left(E_{\text{in}} - E_{\text{out}}\right) + RT \ln\left(\frac{[ATP]\left[H_{\text{in}}^+\right]^{(n-2)}}{[ADP][Pi]\left[H_{out}^+\right]^n}\right) \tag{16}$$

In the equilibrium, when $\mu_{reaction}$ equals zero, we get

$$\Delta\mu_{reaction}^0 = n F z_{H^+}\left(E_{\text{out}} - E_{\text{in}}\right) - RT \ln\left(\frac{[ATP]\left[H_{\text{in}}^+\right]^{(n-2)}}{[ADP][Pi]\left[H_{out}^+\right]^n}\right) \tag{16}$$

$$\begin{aligned} &= n F z_{H^+}\left(E_{\text{out}} - E_{\text{in}}\right) - RT \ln\left(\frac{[ATP]}{[ADP][Pi]}\right) - RT \ln\left(\frac{\left[H_{\text{in}}^+\right]^{(n-2)}}{\left[H_{out}^+\right]^n}\right) \\ &= n F z_{H^+}\left(E_{\text{out}} - E_{\text{in}}\right) - RT \ln\left(\frac{[ATP]}{[ADP][Pi]}\right) - 2.303\,RT\left(n\,pH_{out} - (n-2)\,pH_{\text{in}}\right) \\ &= n F z_{H^+}\Delta E - RT \ln\left(\frac{[ATP]}{[ADP][Pi]}\right) - 2.303\,RT\,n\,\Delta pH - 2 \times 2.303\,RT\left(pH_{\text{in}}\right) \end{aligned}$$

, where $\Delta pH = pH_{out} - pH_{in}$ and $\Delta E = E_{out} - E_{in}$. In the absence of a membrane, the corresponding equation would necessarily lack the electric and ΔpH components and would instead reduce to

$$\Delta\mu^0 = -RT \ln\left(\frac{[ATP]}{[ADP][Pi]}\right) - 2 \times 2.303\,RT\,pH \tag{17}$$

We thus get the following expressions for the [ATP]/[ADP][Pi] ratios in homogeneous solution:

$$\frac{[ATP]_{homogeneous}}{[ADP]_{homogeneous}[Pi]_{homogeneous}} = \exp\left(\frac{\Delta\mu^0 + 2 \times 2.303\,RT\,pH}{-RT}\right) \tag{18}$$

and in the membrane system:

$$\frac{[ATP]_{membrane\,system}}{[ADP]_{membrane\,system}[Pi]_{membrane\,system}} = \exp\left(\frac{\Delta\mu^0 + 2.303\,n\,RT\,\Delta pH + 2\times 2.303\,RT\left(pH_{in}\right) - n\,F\,z_{H^+}\Delta}{-RT}\right. \quad (19)$$

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These ratios are related to each other in the following way:

$$\frac{ratio_{membrane}}{ratio_{homogeneous}} = e^{\left(\frac{\Delta\mu^0 + 2\times 2.303\,RT\,pH}{-RT} - \frac{\Delta\mu^0 + 2.303\,nRT\,\Delta pH + 2\times 2.303\,RT\left(pH_{in}\right) - n\,F\,z_{H^+}\Delta E}{-RT}\right)} \quad (20)$$

$$= e^{\left(-\frac{2.303\,nRT\,\Delta pH - n\,F\,z_{H^+}\Delta E}{RT}\right)} = e^{\left(-2.303\,n\,\Delta pH + \frac{n\,F\,z_{H^+}\Delta E}{RT}\right)} = 10^{\left(-n\,\Delta pH + \frac{n\,F\,z_{H^+}\Delta E}{2.303\times RT}\right)}$$

It is thus apparent that the presence of the pH gradient and the potential difference changes the equilibrium ratio for the ATP → ADP+ Pi half-reaction by the exact same factor postulated by Mitchell in his unrigorous derivation. Since the factor present in exponent is the proton-motive force expressed in “equivalent ΔpH units”, there is no basis to claim, has in [26] , that “it is not possible to derive Δp=ΔE -60ΔpH which is Mitchell's central protonmotive force equation, Eq. (15) in (Mitchell,1966)”.
Other criticisms of the possibility of chemiosmosis in aerobic respiration have been put forward by Manoj[28–30]. The most important arguments seem to be the following:
a) the small number of H_3O^+ in the matrix presents an allegedly “insurmountable” obstacle to the extrusion of a significant number of protons from the matrix and the return of enough protons through the ATP synthase to explain the observed ATP synthesis[28]. However, the calculations above(Figure 2) show that water auto-protolysis can provide as many protons as needed: no matter how many protons return to the matrix through the ATP synthase, the observed pH difference entails that even more protons have previously been taken from the matrix to the intermembrane space.
b) thermodynamic considerations supposedly prevent useful work from being generated by moving protons in and out of the matrix[28]. This arguments would be valid if no energy source were available, but in a functioning electron-transport chain the electron-transfer itself is a spontaneous process which releases the required free energy. The full process can be described through the following thermodynamic cycle:

$$\begin{array}{ccccc}
Electron-donor_{reduced} + electron-acceptor_{oxidized} & \rightarrow & Electron-donor_{oxidized} + electron-acceptor_{reduced} & & \Delta G = -nF\,\Delta E \\
n\,H^+_{in} & \rightarrow & n\,H^+_{out} & & \Delta G = +x\,kcal/mol \\
n\,H^+_{out} & \rightarrow & n\,H^+_{in} & & \Delta G = -x\,kcal/mol \\
ADP + Pi + 2H^+ & \rightarrow & ATP + H_2O & & \Delta G = 7.3\,kcal/mol
\end{array}$$

The full process is depicted below

$$Electron-donor_{reduced} + electron-acceptor_{oxidized} + ADP + Pi + 2H^+ \rightarrow Electron-donor_{oxidized} + electron-acceptor_{reduced} + ATP + H_2O$$
$$\Delta G = 7.3\,kcal/mol - nF\,\Delta E$$

and is clearly spontaneous provided that nFΔE exceeds 7.3 kcal/mol, i.e. if ΔE exceeds 0.16 V for a two-electron process.
c) the number of pumped protons per complex is inconsistent with the overall spontaneity of the process[28]. No sources for the values used (whether proton pumping cost or nunber of protons trasnfered from the matrix) were provided in that publication. A quick estimate (based on a proton-motive force of 200 mV[31]) yields instead a total cost of 36.9 kcal/mol for the extrusion of four protons per electron pair of complex I[32], and two protons per electron pair by each of complex III[33] and complex IV[34], which can be easily provided by the transport of two electrons from

NADH to O_2 (1.13 V, i.e. 52 kcal/mol). There seems to be, therefore, no reason to suspect of any thermodynamic discrepancies, even if the actual proton-pumping numbers are wrong by as much as 40%.
d) the electron-transfer moities in complex I are not aligned in a sequence of strictly increasing redox potentials, which is argued to prevent efficient electron-transfer. This argumentation does not take into account the observations that ocasional endergonic steps in an electron-tunneling chain are not impeditive of efficient electron transport, and that the proximity (<14 Å) of the electron-transporting components in these chains renders them generally robust to changes in reorganization energy, endergonicity and packing density of the intervening protein atoms[35].
e) the spontaneity of ATP hydrolysis is argued to imply that ATP synthase can only function as a ATP hydrolase. This is indeed the observed reaction in the absence of a proton-motive force[36], but this observation, *per se*, does not entail that the same must be observed in the presence of a proton gradient. Indeed, abundant experimental evidence[24] shows that in isolated, energized liposomes containing ATP synthase and no other electron-transport chain component, the existence of a proton motive force leads to ATP synthesis that can not be ascribed to any source other than the ATP synthase itself. Futher details of this author's argument also rely on the unsubstantiated claim that the ATP synthase has a higher affinity to ATP than to ADP or Pi, whereas the opposite has been experimentally confirmed[37].

As an alternative to chemiosmosis, Manoj *et al.* suggest that the electron-transport chain instead uses the electrons to generate reactive oxygen species[30], and that these drive the formation of ATP from ADP and Pi without the intervention of ATP synthase in a process they call "murburn". As confirmation, they show the results of an experiment where 2-50 µL of a solution of 20 mg of KO_2 (diluted in an undisclosed amount of DMSO), was added to a solution of 200 mM ADP, 10 mM Pi and 1 mM $MgCl_2$. The report is extremely short in experimental detail: no control using only ADP and superoxide was performed, nor was the concentration of the KO_2 solution mentioned, which detracts from its reliability. They observed that, after 5 min, addition of luciferase led to luminescence equivalent to that expected from the presence of approximately 1 µM ATP and concluded that their model was indeed vindicated. The extremely low yields of ATP obtained with such high concentrations of ADP (vs. the actual 50 µM present in cells[38]) , however, strongly argue against any possible physiological role of this reaction even if it indeed is real, rather than an experimental artifact. As additional arguments for their claims against the current chemiosmotic paradigm, Manoj further argues[30] that "conclusive" evidence comes from the lack of ATP synthase in certain stages of the *Plasmodium* life cycle and from the ability of ATP-defective *E. coli* to grow, albeit at a lower rate. Neither of these arguments is actually "conclusive", since it is well known that glycolysis alone (followed by fermentation) can produce enough ATP for the growth of fermentative organisms[39] and that in the blood stage of the Plasmodium life cycle energy is provided by glycolysis (folowed by NAD^+ regeneration through fermentation) and residual electron flow through the electron-transport chain is used to provide electrons for dihydroorotate dehydrogenase instead[40] . In other stages of the parasite life cycle, ATP synthase is indeed operative and indispensable[41]
Beyond the experimental and thermodynamic arguments presented above, there are also strong evolutionary and comparative physiology arguments against the replacement of the chemiosmotic model by the "murburn" hypothesis. For example, why would a membrane-bound system be invariably necessary for coupling reduction of the external electron acceptor to ATP synthesis if only oxygen radicals were needed to poer ADP phosphorylation? Moreover, membrane-bound electron-transport chains are ubiquitous throughout all realms of life, including in strictly anaerobic organisms. If chemiosmosis were indeed "impossible" and ATP synthesis were performed through the intervention of radical oxygen species, how could oxidative phosphorylation be performed during anaerobic respiration and different growth yields from the same nutrient be obtained only by changing the oxidation state of the respiratory acceptor[42]? Similarly, an electron-transport chain powered by choromophore excitation is crucial for the production of ATP for carbon production

during photosynthesis in both oxygenic and anoxygenic photosynthetic organisms. Since the first anoxygenic photosynthetic organisms evolved far before the appearance of atmospheric O_2[43–45], the correctness of the “murburn” scheme would imply that those organisms would have been incapable of converting light energy into ATP and therefore their light-sensitive reaction centers would only provide excited electrons for the generation of NADH/NADPH, requiring those organisms to obtain the ATP necessary to fixate CO_2 through other means. Apart from light, the only other source of energy for autotrophs is the oxidation of inorganic reactions compounds such as iron[46] or ammonia[47], which requires electron-flow towards membrane-bound terminal oxidases and (once again) chemiosmotic ATP synthesis. It is thus apparent that, even were one to accept that the “murburn” scheme could be operative in aerobic conditions, an extremely vast landscape of metabolic diversity would still require a different explanation for the ATP synthesis mechanism. Chemiosmosis, in contrast, offers a unifying framework, buttressed by decades of solid experimental support and theoretical foundation. It may eventually be found wanting, but parsimony and intelectual humility should force us to postpone its replacement to a moment when enough shortcomings have been found and a novel paradigm which offers at least as much explanatory power is ready. None of the proposals discussed above show either such shortcomings in chemiosmosis nor any suitable first-step towards such a novel theory .

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