This is a pre-review version of the paper published as

“Response to “Molecular-level understanding of biological energy coupling and transduction: Response to “Chemiosmotic misunderstandings” ””
in Biophysical Chemistry.

The version of record can be found at
https://dx.doi.org/10.1016/j.bpc.2020.106512

Response to “Molecular-level understanding of biological energy coupling and transduction: Response to “Chemiosmotic misunderstandings” ”

Pedro J. Silva[1,2]
[1]FP-ENAS/Fac. de Ciências da Saúde, Universidade Fernando Pessoa, Porto, Portugal
[2]UCIBIO@REQUIMTE, BioSIM, Departamento de Biomedicina, Faculdade de Medicina, Universidade do Porto, Porto, Portugal

keywords: Bioenergetics, Chemiosmosis, Gauss’s law, proton-motive force

Abstract

The most recent contribution by Sunil Nath in these pages is, mostly, a repetition of his previous claims regarding failures of the chemiosmotic hypotheses, supplemented with some fresh misunderstandings of the points I had sought to clarify in my previous critique. Considerable portions rehash 50-60 years-old controversies, with no apparent understanding that the current chemiosmotic hypothesis, while birthed by Mitchell, differs from Mitchell's details in many respects. As such, Nath has devoted much time dealing with a few errors (or wrong hypotheses) by Mitchell (in a few places I would almost venture to say "typographical mistakes in typesetting") and presents the ensuing conclusions as "refutations" of the chemiosmotic paradigm, completely neglecting that such details (such as the precise H^+/ATP or H^+:O ratios) are completely irrelevant to the reality (or not) of an electron-transport chain that uses the free energy liberated by electron-transfer to remove H^+ from a compartment, to which it returns through and ATP synthase which uses the energy in that spontaneous return to drive ATP synthesis. The thermodynamical mistakes and misunderstandings of the relevant literature present in Nath’s new contribution are so numerous, though, that I feel forced to call the attention of the readers of “Biophysical Chemistry” to them.

Text

Sunil Nath’s response[1] to my critique[2] of his work touches upon a large variety of topics. A full response, therefore, requires the systematic overview of each of the sections in his recent paper, aiming at clarifying several misunderstandings and misstatements present therein.
The first two sections of Nath’s contribution recall the details of 50-60 years old controversies regarding chemiosmosis. Although much of that history has already been described profusely in several works by Marcel Weber, John Prebble, Michel Morange and Bruce Weber [3–10], S. Nath again recalls, for example, the opposition of Lehninger, Slater and R.J.P. Williams to the

chemiosmotic hypothesis. The importance of the objections of those practitioners does not reside in their eminence, however, but on the fundamental logic of their arguments. The relevance of Nath's arguments against the chemiosmotic hypotheses is independent of the existence (or absence) of any other challenges to that hypotheses, and (conversely) the existence of previous objections (no matter how eminent their proponents) is irrelevant for the correctness of Nath's arguments. However, if Slater's, Lehninger's and Williams' opinions have any relevance for the present paper, one should also look at whether or not they changed their mind (since, *ex hypothesis*, they should be the best judges of the value of their objections). A proper reading of the literature, however, reveals that E.C. Slater, Lehninger and R.J.P. Williams (even if still harboring objections to several details) did become convinced of the overall correctness of the fundamental insights of the chemiosmotic hypothesis. For, example, Lehninger's publications in 1976-77 [11,12] do observe proton ejection accompanying electron-transfer and ATP synthesis upon proton return to the matrix, even if they disagree with the precise number of protons postulated by Mitchell. The specific H^+/O/ATP stoichiometry postulated by Mitchell is not, contrary to Nath's claims, in any way crucial to the chemiosmotic hypothesis, as can be easily checked by its conspicous absence in any standard textbook in Biochemistry. The currently accepted stoichiometries (four protons per electron pair of complex I[13], and two protons per electron pair by each of complex III[14] and complex IV[15]) are completely plausible in thermodynamic grounds, as can be easily checked by comparing the magnitude of the energy released by electron transfer from NADH to O_2:

$E^{o'}$ (NAD^+/NADH)= -0.32 V

$E^{o'}$(O_2/H_2O) =0.81 V

In mitochondria, NAD^+/NADH ratio is around 8[16] , and the partial pressure of O_2 is around 30-40 mmHg[17].

The actual potentials are therefore:

E (NAD^+/NADH)= -0.32 V + RT/2F ln [NAD^+]([H^+]/10^{-7})/[NADH] = -0.323 V (at pH 7.8)

$E^{o'}$(O_2/H_2O) =0.81 +RT/4F ln pO_2 ([H^+]/10^{-7})^4 = 0.730 V (at pH 7.8)

(The use of [H^+]/10^{-7} instead of plain [H^+] is needed because the quoted standard potentials are $E^{0'}$ instead of E^0, and therefore use a 10^{-7} M as standard concentration for H^+, instead of the 1 mol/L used as standard for all species)

The energy released when 2 electrons are transferred from NADH to O_2 is therefore

$\Delta G = -2F\Delta E = -2 * 96485$ C/mol /4184 J/kcal *1.05 V= -48.6 kcal/mol

Assuming that the protons must be ejected against a pmf of 200 mV (in the upper range of currently accepted values[18], and therefore the least convenient for my argument), this amount of energy is enough to expel 10.5 protons per NADH, which can perfectly accommodate the currently accepted stoichiometries (4 protons per electron pair in complex I, 2 in complex III and 2 in complex IV). This energy is also enough to synthesize at least 3 ATP per NADH, whether one uses the commonly accepted ΔG of ATP synthesis under physiological conditions (around 13 kcal/mol) or the less favourable one (15.6 kcal/mol) measured by Cockrell *et al.[19]* and preferred by Slater[20] in his 1967 evaluation of the chemiosmotic hypothesis.

The later writings of E.C. Slater[21,22] show that he eventually accepted large portions of the chemiosmotic hypotheses (even if he still preferred a collisional model[23] between electron-transfer-proteins and ATP synthase as the most plausible form of proton transfer from the former to the latter). For example, from reference [12] (all emphases mine): "More fundamentally, there are still workers in the field who believe that there is a more direct interaction between the redox enzymes and the ATPase, probably via protons (36), than envisaged by the chemiosmotic hypothesis. The jury is still out on this point. **However that may be, the chemiosmotic theory, from being 'an elegant but outside alternative' (3) in 1961, has now become the accepted mechanism of oxidative and photosynthetic phosphorylation, taught in all elementary classes of biochemistry. Proton pumps driven by light energy or the hydrolysis of ATP have been found in many types of prokaryote and eukaryote cells. Whole symposia are devoted to vacuolar-type, proton-motive ATPases. The three-dimensional structures of bacteriorhodopsin, the light-driven proton pump, and the bacterial photosynthetic reaction centre, a typical electron-carrying arm of a Mitchell loop, have been elucidated and are fully in accord with the principles of the chemiosmotic theory.**" and from reference [13] "The postulate of a non-phosphorylated high-energy intermediate in the chemical hypothesis is correct; its nature is not. **The postulate of the chemiosmotic hypothesis that this intermediate is an electrochemical proton gradient is correct**; the way in which it was thought to synthesize ATP is not. The binding-change mechanism of ATP synthesis proposed by Boyer is probably correct; but will the rotary mechanism depicted in the textbooks survive challenges?"

The position of R.J.P Williams is also much more nuanced than S. Nath seems to believe: Williams did voice objections to a chemiosmotic model with delocalized protons[24], but a full reading of the record shows that Williams vehemently faulted Mitchell for allegedly using his insights without attribution[25,26] (which would have been a strange thing to do indeed, if Williams had felt the chemiosmotic hypothesis to be completely wrong).

S. Nath then claims that "**Silva appears to believe that modification of 'a few of the initial details' of the chemiosmotic theory is inconsequential [18]; however, even these presumed minor aspects are sufficient to destroy the chemiosmotic theory. He mentions two points: (i) the "specific H + /ATP ratio proposed," and (ii) "full attribution" to “pH across the membrane" [18]. I take them one by one.**"

In contrast to the author's claims, no modern description of the chemiosmotic hypothesis (such as can be found in any number of Biochemistry textbooks, or on Raymond Chang's Physical Chemistry[28]) relies on the precise H^+/ATP ratio postulated by Mitchell or requires that the full proton-motive force is due to the intervention of protons (rather than having a sizeable portion due to a pre-existing electric potential difference across the membrane). Currently accepted H^+ / ATP and H^+/O ratios are actually fully consistent with the relevant thermodynamics (as shown above and in my previous paper). Whether Mitchell accepted or not a specific H^+/ATP ratio "until his dying day" is irrelevant for the present understanding of the theory, just as the present acceptance of heliocentrism and Earth's rotation does not depend either on the existence of sun-centered circular orbits and epicycles (as proposed by Copernicus) or on Galileo's wrong attempt to explain tides through Earth's rotation around its own axis. Discrepancies in H^+/ATP measured with rudimentary 1960’s technology also do not seem to me to be a strong argument against (or for) one hypotheses or the other, since currently measured values are expected to be much more accurate than the ones obtained with 1960's technology.

Nath then continues "**My fundamental objection that Mitchell's Eq. (10) in the mathematical framework of the chemiosmotic theory [30] is wrong and implies that ΔE is zero within the framework of the mathematical equations of the chemiosmotic theory.**"

Mitchell’s eq. (10), as printed in his 1966 Glyn report[29], is indeed wrong, although I believe that is a case of typographical mistakes and confusing notation. Regardless, Mitchell himself, in his self-published 1968 Glynn Report[30], presented a fresh derivation of the proton-motive force in the context of a delocalized potential (equations 33-39, in pages 23 and 24) which is devoid of errors (or typographical mistakes) and is identical to the one that can be easily found in standard Bioenergetics or Physical Chemistry textbooks[28,31]. That derivation comes directly from the definition of electrochemical potential of any species "X" (here depicted as "μX"):

$\mu X = \mu X(\text{standard conditions}) + RT \ln [X] + z F E$ (where z is the charge of the species, F is the faraday constant, E is the electric potential and other symbols have their usual meanings)

In the case of a membrane separating different amounts of H^+ on the “outside” (out) from its "inside" (in), we get for the electrochemical potential on the inside:

1) $\mu H^+_{in} = \mu H^+(\text{standard conditions}) + RT \ln [H+]_{in} + F E_{in}$

and on the outside:

2) $\mu H^+_{out} = \mu H^+(\text{standard conditions}) + RT \ln [H+]_{out} + F E_{out}$

Subtracting expression 2 from expression 1, we get :

$\mu H^+_{in} - \mu H^+_{out} = RT \ln [H+]_{in} / [H+]_{out} + F [E_{in} - E_{out}]$ <=>
$\mu H^+_{in} - \mu H^+_{out} = RT \ln 10 \times \log_{10} [H+]_{in} / [H+]_{out} + F [E_{in} - E_{out}]$ <=>

From this, one can easily write (from the definition of pH as -log $[H^+]$)
$\mu H^+_{in} - \mu H^+_{out} = RT \ln 10 \times (pH_{out} - pH_{in}) + F [E_{in} - E_{out}]$

and therefore, by defining ΔpH as $pH_{in} - pH_{out}$ and ΔE as $E_{in} - E_{out}$
$(\mu H^+_{in} - \mu H^+_{out}) = F \Delta E - (RT \ln 10) \Delta pH$ <=>
$(\mu H^+_{in} - \mu H^+_{out}) / F = \Delta E - (RT \ln 10 / F) \Delta pH$

By defining the proton-motive force as $(\mu H^+_R - \mu H^+_L) / F$, we get the familiar expression pmf = $\Delta E - (RT \ln 10 / F) \Delta pH$, which is the one I also obtained using a thermochemical cycle and that S. Nath claims cannot be deduced in the presence of a delocalized potential. His insistence is all the more surprising because Nath’s papers show that he is acquainted with Mitchell’s 1968 Glynn report, and he cannot therefore ignore that Mitchell’s flawed (or mistyped) deduction in the earlier 1966 Glynn report was superseded by that in the 1968 repost and is NOT the current basis for the acceptance of the equation for the proton-motive force.

S. Nath then contends with my description of Fig. 1A in my paper[2] (a precise graphical representation of the non-conducting sphere model used in section 4 of Nath's paper "Modern

theory of energy coupling and ATP synthesis. Violation of Gauss' law by the chemiosmotic theory and validation of the two-ion theory") as “Nath's model”. Contrary to Nath’s assertions, no confusion with Nath's two-ion model of ATP synthase can be envisioned here, since that model is not mentioned in my paper at all. On the contrary, the only mentions of the words “Nath” and “model” in close proximity in the paper which is the target of Nath’s critique are the following: “[Nath’s] **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 (Fig. 1A). [….] Figure 1: Comparison of the Nath model (A) and the correct model (B) of charge distribution on a model spherical mitochondrion.**” Nath also claims that the model depicted in Fig. 1A is originally Mitchell's, and not Nath’s. This is also a misunderstanding: Mitchell’s 1968 Glynn report[30], which Nath cites often, clearly states (p. 53) “**[...] systems where the inside of the vesicle is negatively charged (intact mitochondria and intact bacteria)[...]**”, and therefore no model which contains the positive charges on the inside (like the one used by Nath in section 4 of the paper I critiqued[32]) can be described either as “Mitchell’s model” or as a model of the charge distribution accepted by modern Biochemists. Indeed, since cells are macroscopically electro-neutral, every compartment which expels protons to the cytoplasm must retain enough excess negative charges to balance them, otherwise electrical charge would be created *ex nihilo*. Mitchell clearly stated this throughout his ouevre: he repeatedly talks of “displaced charge” (rather than “newly-created” charges), which can only be interpreted as stating that the total charge of the complete system at the end of proton ejection from mitochondria is the same as that in the beginning, but is distributed in a different way. The only places where Mitchell speaks of positively-charged vesicles are “**sonic mitochondrial particles, chromatophores and chloroplast grana**” (also on p.53 of that publication). The thylakoid lumen is known to be topologically equivalent to the mitochondrial inter-membrane space, and sonicated mitochondrial particles (besides being artificial constructs with no physiological role) are known to be topologically inverted relative to the physiological situation (pages 167 to 170 of [29], and references therein).

Other errors are evident in this section. Nath states “**The very low electrical potential of 86 mV calculated by Mitchell for the system could only have been obtained by violation of Gauss’s law [84,85]. Correct application of the law led to the large potential of 86 × 10^8 mV to maintain een such a small departure from bulk electrical neutrality in the system**”. Analysis of Nath’s paper, however, shows that the large value he obtained depended on the use of a model mitochondrion with **1 cm radius** (I quote “this phase was taken to contain an excess of 10^{-10} mole

of a monovalent ion. Using the expression for the electrical potential, V of a charged sphere of radius r in vacuo, V]Q/(4πε0r), he obtained V = 0.96 × 10^{-5}/(1.11 × 10^{-10}×**10^{-2}**) = 0.86 × 10^{7} V, a phe- nomenally large electrostatic potential. ”[32]) Later in his paper, Nath objects to my computations by arguing that my mitochondrion model is “just a sphere. There are no details of internal organelle structure, no mention even of cristae, no channels[…]”. I honestly cannot understand how Nath can claim that *his* use of a spherical mitochondrion without cristae with a **1 cm-radius** (which would make it far larger than the largest human cells) and positive charges in the inside is enough to refute the chemiosmotic hypotheses, and then argue that *my* model is “incomplete and inadequate” (in spite of having a radius compatible with the measured volume of mitochondria and the correct charge distribution implied by the chemiosmotic theory) because my model (like his own) lacks cristae!

Nath then claims that “**Mitchell finally settled on a smaller sphere of 1 µm radius to model a mitochondrion , instead of a sphere of 1 cm radius, so the electrical potential for the above small net charge imbalance of 1 × 10^{-10} mol would work out to be 86 × 10^{12} mV by Gauss’s law. Mitchell has subsequently assumed a measurable physiological charge separation of 1 × 10^{-3} mol of ions [...]**”. This is also wrong: in Mitchell’s 1968 report (page 26) Mitchell set out to prove that the charge imbalances postulated by his theory are consistent with the lack of extremely large potential differences between both sides of the membrane. He neglected the negative charges inside the mitochondira in order to be able to apply the formulas for the potential arising from a shell of positive charges (taken from Guggenheim). He used a charge density of 10^{-10} mol positive charge in a spherical shell with 1 cm radius. These values were not chosen at random: Mitchell’s previous computations of charge imbalance using the known membrane capacitances (section 5.1 in Glynn report 1966[33]) yield the following charge density:

capacitance = 1 µC/V/cm^2= 10^{-6} C * 96485 C/mol / V / cm^2 =1.03 ×10^{-11} mol/V /cm^2

for a 200 mV difference of potential, this yields

charge imbalance around the mitochondria = 2.06 ×10^{-12} mol of univalent ions/cm^2

The surface of a sphere with radius 1cm is 4 π $(1\ cm)^2$ = 12.566 cm^2. The charge density of the model that Mitchell used was therefore 10^{-10} mol /12.566 cm^2 = 7.96 ×10^{-12} mol / cm^2 (and therefore about 4 times larger than the charge density around the mitochondria). Clearly, if this charge density did not yield excessive electrical potentials, then neither would the charge density around mitochondria. For a 1 cm sphere, the potential is 0.86 ×10^{7} V (rather than the 0.95×10^{7} V written by

Mitchell in Glynn report 1968, due to the lack of proofreading in those self-published texts). Since mitochondria do not have 1 cm radius, Mitchell therefore attempted to computed the potential difference for a sphere 10^4 times smaller (i.e. 10^{-6} m radius). The charge used for the new computation obviously cannot be, like Nath used in his papers, the same as the charge around the 1-cm radius model: it should be the charge that yields a charge density consistent with the experimental measure of capacitance, and therefore is computed by charge=charge density× area. Since the radius is now 10^4 times smaller, the surface is 10^8 smaller and the total charge is therefore 10^{-18} mol and Guggenheim's formula yields

potential= 10^{-18} mol × 96485 C/mol / 1.11 ×10^{-10} / 10^{-6} = 869 V.

Mitchell indeed made a mistake here, but not the one that Nath argues he did, and of a significantly smaller (though still too large) magnitude: instead of computing the charge around the smaller model using the charge density computed from the experimental capacitances, Mitchell wrongly considered that when the whole charge was divided throughout a given number of spheres with 10^{-6} m radius, he should have a number of new spheres whose volume was equal to that of the original large sphere (rather than a number of spheres with surface area equal to the original one). This error will naturally yield, however, (from the capacitance expression $C=\Delta Q/\Delta V$) a model where the charge imbalance is quite smaller that the one implied by the 200 mV pmf difference. Mitchell therefore obtained a number (87 mV) which was 10^4 times smaller than the one he should have obtained had he made the computation correctly, and since that value was already low enough, he stopped his analysis without the additional step of including the contribution of the inner negative charges, since that could only further decrease the potential and he had already (though mistakenly) "proved" that no exaggerated potentials would arise from the charge imbalances. I agree that this was a grave error by Mitchell, and I can only deplore that his Glynn reports from 1966 and 1968 were self-published instead of peer-reviewed: the fresh eyes of an attentive peer-reviewer would have pointed out these and other issues prior to their publication, and avoid the subsequent confusion. Contrary to Nath's claims, I could also not find, in Mitchell's Glynn report from 1968[30] any mention of him assuming "a measurable physiological charge separation of 1×10^{-3} mol of ions" in a mitochondrion, which (besides grossly disagreeing with the measured membrane capacitances) is many orders of magnitude above any charge separation used by Mitchell in other works (e.g. 63 ions per μm^2 in [33]). The closest I could find to a " 1×10^{-3} mol of ions" in his writings was a computation (in p. 104 of his 1966 Glynn report [29]) of 0.8 mM **concentration** of negative charge "left-behind" in a mitochondrion when 12500/μm^2 protons are ejected (to yield a 200 mV difference using the experimental value for the capacitance).

Regardless of Mitchell's faults, we can now proceed from the analysis obtained from the proper application of Gauss' law to a model with the appropriate charge distribution, which I performed[2] after being prompted by Nath's analysis. This latest Nath's contribution does not dispute the correctness of my derivation, but only its lack of topological features needed for a full model of a mitochondrion. The neglect of cristae from my model (as well as Nath's[32]) was needed because the mathematical application of Gauss' law requires models with very high symmetry (infinite planes, infinite cylinders or spheres) where the field is homogeneous across the whole Gauss surface. However, rigorous physics-based descriptions of the influence of cristae (by far better physicists than me) are available in the literature[34] and amount to only a 30 mV drop between the potential at the inner boundary membrane and the cristae, and therefore the neglect of cristae in my model should have a very modest effect on the final result.

Nath also states "**The model in Fig. 1B of ref. [18] does not explain how a delocalized potential, ΔE is created and how it is maintained. In a field of this polarity created by electron-coupled proton translocation, protons and 'positively-charged species present in this set-up will tend to move to the inside of the mitochondrion** "
Any attempt to argue against a delocalized potential in mitochondria using the arguments used above by Nath would apply equally well to the electric potentials in neuron membranes. In mitochocndria, the potential is known to be maintained (*inter alia*) by proton pumping through the electron transfer chain and (as in any membrane-bound system which contains non-diffusible charged macromolecules) by Donnan effects, as can be checked in the section on electrochemical potentials in any Physical Chemistry textbook. It is also very likely that a substantial part of it arises from different permeabilities of the membrane to different ions (as is the common explanation for the membrane potential in neuronal cytoplasmic membranes according to the Goldman-Hodgkin-Katz equation). Under the influence of the potential, protons obviously tend to move to the mitochondrial matrix (and anions would tend to move out) but the potential nonetheless does not collapse because the inner membrane is not very permeable to ions, except at specific channels such as those provided by uncoupling proteins and ATP synthase: in a mitochondrion treated with external ionophores, ATP synthesis decreases markedly because most of the proton gradient now has additional ways (the added protonophores) to re-enter the matrix: again, this is explained in standard textbooks.

In section 4, Nath attempts to use Gauss' law to obtain the field across a half-channel with a single charge in its interior. The expression he obtains, however, can clearly be seen to be wrong because Gauss' law and Coulomb's law cannot disagree, as can be checked in every introductory text on

electromagnetism or even in general Physics texts (as in the 4th chapter of the second volume of Feynman's Lectures on Physics[35]): in fact, Gauss' law reduces to Coulomb's law when only a point charge is present (as in the model presented here by Nath) and, conversely, Gauss' law can be deduced from Coulomb's law if fields arising from different static point charges are additive. In the half-channel model used by Nath, which contains a single point-charge, direct computation of the field from Coulomb's law is trivial:

Field= q/ 4πεr

This is obviously different from the result that Nath got in his eqs. 13 and 14. Since Coulomb's law cannot disagree with Gauss' law, this means that an error was introduced in the surface integral computation: indeed, one can only multiply (like Nath did) the flux by the surface area if the flux is absolutely equal in every point of the surface, and that is not the case in this model. Besides, I cannot understand why Nath focusses on the magnitude of the electrical field strength (which he fails to state where it was computed. One can easily obtain any given value between zero and infinity just by choosing an appropriate *r*, after all) instead of the potential differences, which can indeed be compared with the experimental values. Even if the computation of the field were correct, I cannot understand the point of his comparison of the field in a 2 nm half-channel with the field in the inside of a mitochondrial membrane, or how the comparison of those values has anything to do with the validity of the computations I performed in the paper that Nath is criticizing.

In section 5, Nath states "**it has never been shown by experiments that protons translocate through the a-subunit access channels.** " Plenty of experiments have shown proton translocation through ATP synthase (e.g. Paola Turina, Jagendorf, even Lehninger 1976-1977, quoted above). Whether or not that occurs in the a-subunit or through some other hitherto unsuspected subunit is, however, irrelevant: in either case, a combined pH/electrical proton-motive-force would still be responsible for powering the ATP synthase. Current data, in contrast to Nath's representations, do strongly point to the involvement of the a-subunit there, such as it containing two half-channels that meet the c-ring at the exact spot where a c-ring Glu residue can become protonated/deprotonated (and which, after rotation of the c-ring, is placed in the correct position to transfer the proton to the other half-channel). Unlike Nath contends, there are as yet no incontrovertible data showing anion-translocation through the a-subunit either: the half-channels are quite narrow and (more importantly) they they do not provide a continuous surface for a large molecule to flow through from one end to the other. Positing that they transport anions implies that the c-ring must receive an anion from the end of one of the half-channels and feed it to the

beginning of the other half-channel, and the presence of a Glu residue (which can either be negatively-charged or neutral, but not positively-charged) at the relevant position in the c-ring argues against the binding of an anionic species there. This can be checked any interested reader just by looking at the relevant structures (for example, 7JG5[36] or others [36–38]) and seeing that Glu65 (the key residue postulated to transfer the proton from one half-channel to the other) of three of the c-subunits are placed under 5 Å away from the a-subunit (Figure 1). The author's confident claims otherwise are therefore incomprehensible to me. Nath also seems to believe that the inhibition of ATP synthase by tributyltin chloride supports the involvement of anion translocation. Tributyltin, however, is not an anion and is instead known to bind strongly to Cys residues. Inhibition of ATP synthase by tributyltin therefore simply means that a mechanistically-relevant portion of ATP synthase contains a solvent-exposed Cys residue. The generalization attempted by the author (that the inhibition of some anion transporters by tributyltin means that every instance of inhibition by tributyltin points to the relevant enzyme being an anion trasport) is logically unwarranted: “a implies b” can be disproven by a single observation of “a” and “NOT b”, as any introduction to logic will show[37]. That generalization (in what regards tributyltin inhibition) has been experimentally disproven by the inhibition of 11β-hydroxysteroid dehydrogenase by tributyltin [38] and by the existence, in the PDB, of two instances of proteins inhibited by tributyltin bound to them: PPAR gamma[39] (PDB: 3WJ4) and RXRalpha[40] (PDB:3E94), none of which is an anion channel, but rather a nuclear receptor. Therefore, the same logic that states that tributyltin inhibition shows that a protein is an anion channel, could likewise be used to argue for the ludicrous contention that every protein inhibited by tributyltin is a nuclear receptor or a steroid dehydrogenase (and that therefore the observation of ATP synthase inhibition by tributyltin would mean that the ATP synthase would also be a nuclear receptor or a steroid dehydrogenase).

In section 6, author states "**Silva has applied the equilibrium condition Δμ= 0 (15) to analyze the ATP reaction. However, it is important to use Eq. (15) appropriately. The equilibrium condition of Eq. (15) can only be applied to a given species or ion if the system exhibits a continuous region through which the molecule or ion can move freely[...] If the bulk aqueous phases are not in direct contact or if there are no connected regions, as in all rotary or alternating access transporter models, nothing can be said about the chemical or electrochemical potential of a molecule or ion, whether it is greater, lesser, or equal to the potential on the other side of a membrane**"

This is incorrect: protons bind to the intermembrane-space-facing portion of the proton channels because their binding to those sites is favourable (i.e. their electrochemical potential in those sites is

more favorable than in the intermembrane space). When the protons instead face the matrix, they can only unbind if the electrochemical potential of H^+ in the matrix is more favorable than in their current binding sites. Therefore, net movement of protons can only occur if the electrochemical potential in the matrix is more favourable than in the inter-membrane space. The net favourability (or unfavourability) depends on the spontaneity of the reaction, which in turn depends on the Gibbs free energy. G is a state function, and therefore changes in free energy only depend on the initial and final states, and not on the path taken (whether continuous, or step-wise): having a continuous channel or an “oscillating”channel (sometimes open to one side, others open to the other side) is therefore irrelevant for the analysis.

In section 7 , author states "**Another faux pas arising as a consequence of Silva's Eq. (17) [18] is that an electrical field can directly alter the standard state free energy of the ATP reaction and the ATP/(ADP.Pi) ratio. However, such direct effects of an electrical field on a chemical reaction have never been verified, as discussed by Nath in 2003 [pp. 146-147 of ref. 11]. In his last paper on the subject, Williams specifically stated that Mitchell's mechanism of ATP synthesis was electrolytic-field driven and is impossible [58].** "
Chemiosmotic effects are widely accepted (as can be checked in any Biochemistry textbook) to not depend on field-derived effects, but on conformational changes powered by proton movement along its electrochemical potential (a discovery which netted Boyer a well-deserved Nobel Prize). Chemiosmosis is still involved, though: it is the very proton-motive force postulated in chemiosmosis that powers those conformational effects. However much the current chemiosmotic understanding is indebted to Mitchell's insights, the currently accepted theory differs from the details he postulated. Such details (important as they are to our understanding of Nature) do not, however, render the core insights invalid, and any "refutation" of the chemiosmotic theory should refute the current understanding of chemiosmotic theory, not the incipient theory that Mitchell is rightly (or wrongly, according to R.J.P. Williams) credited for, just like any hypothetical "refutation" of heliocentrism published in 2020 should attempt to refute the current celestial mechanics, not the Copernican model of circular orbits and epicycles. This section contains other, important errors, which will be discussed at the end of this review.

In section 10, Nath claims that his “**docking studies on the cryo-EM structures [....] clearly reveal bound succinate in the aqueous cavity of the half-channels at the a-c interface in FO** "
This claim (besides lacking concrete details regarding the size of the docking area used) eludes the well-known limitations of docking approaches: any molecule can be shown to dock to any surface

provided that there is physical room to accommodate it, but that does not mean that (*in vivo*) that binding mode is energetically favored over remaining free in solution[38]. Moreover, the very nature of the quick scoring functions used entails that many of the docking poses found will be artifacts and therefore molecular dynamics simulations in explicit solvent must be performed to confirm a binding site. In most cases, putative "binding sites" present in protein surfaces are therein revealed to be artifacts (especially if the original docking space has been constrained to a small area), and therefore molecular docking is famously insufficient to confirm that a binding site exists (or to discriminate whether a confirmed binding site has any allosteric or catalytic role). In this specific instance, the author would also have to show that the ligand can indeed enter (and leave) the cavity from the opposite side. The issue is not merely a question of ligand size: experimental observation of larger ligands binding to the c-ring/a-ring interface (like bedaquiline) cannot be used to argue for the possibility of succinate transport through that interface because Nath's two-ion model requires not only anion binding but also anion translocation. Bedaquiline, moreover, is not an anion, and therefore it is not clear how its binding close to the Glu postulated to receive the proton should be argued as support for the binding of an anion.

The most troubling errors in Nath's paper begin, however, at the end of section 7. Here, Nath claims "**Finally, the membrane to homogeneous solution ratio of ATP/(ADP.Pi)) in Eq. (21) of Silva's paper [18] is meaningless and cannot be experimentally evaluated because no one has to date achieved ATP synthesis in homogeneous solution. Hence it is inappropriate to use the ATP/(ADP.Pi) ratio in homogeneous solution as a reference. It is only a trick to cancel off the "mathematically troublesome" terms of in the standard state free Gibbs energy, $\Delta\mu^0$ and the 2.303 RT pH_{in} in the equations. With these terms present, the author's derivation would have failed.** ".

No trick was involved here: only a straightforward application of a thermochemical cycle, which is always valid when dealing with state functions, which (by definition) do not depend on the path taken from reactants to products. Thermochemical cycles can be trivially constructed from individual reactions, which I did in my paper that Nath is now criticizing (Figure 2): reaction A ($ADP+Pi+2\ H+ \rightarrow H_2O + ATP$, which I called "ATP synthesis in homogeneous mediium") was added to reaction B ($n\ H^+_{out} \rightarrow n\ H^+_{in}$) to obtain a new reaction C ($ADP+Pi+2\ H^+ + n\ H^+_{out} \rightarrow H_2O + ATP + n\ H^+_{in}$, which I called "ATP synthesis in the membrane system") Since G is a state function, $\Delta G_C = \Delta G_A + \Delta G_B$. From this basic identity it obviously follows that ΔG_B (the energy released by the movement of H^+ from the intermembrane system to the matrix , i.e.. the proton-motiveforce) can be computed as $\Delta G_C - \Delta G_A$, and the computations in equations 12 to 20 of that

paper are simply a derivation of ΔG_B as a function of the equilibrium constants of reactions C and A (which can be trivially computed from ΔG^0=-RT ln K). The grounds of the reasoning in those equations should have been be absolutely obvious to anyone who has ever understood the basic logic of a thermochemical cycle, and any description of that reasoning as “a trick” is therefore unwarranted.

The most striking error in Nath’s paper appears, however, in section 8, which states that “**Silva, however, seems to falsely believe that “water auto-protolysis can provide as many protons as needed. Calorimetric experiments on the energetics of ionization do not validate this latter view** ” and offers as support for the “falsity” of my “belief” the (correct) facts that ΔG^0 and ΔH^0 for water dissociation is very positive. ΔH^0 is irrelevant here: endothermic reactions can indeed be spontaneous, as is well known even to high-school students. The criterion for reaction spontaneity, however, is not exothermicity (a negative change in ΔH, enthalpy) , but exergonicity (a negative change in ΔG, the Gibbs free energy), as described in the Chemical Thermodynamics section of any introductory Physical Chemistry or Biochemistry textbook. Nath’s contention that the high positive value of ΔG^0 prevents auto-protolysis from occurring is an egregious error: as every Thermodynamics and Physical Chemistry textbooks states, standard free energies (ΔG^0) are computed/measured when EVERY intervening species is present at standard conditions (1 mol/dm^3 for solutes, 1 atm for gases, unit activity assumed for solvents). One cannot use standard Gibbs free energies directly in other conditions, but must instead compute
$\Delta G = \Delta G^0$ + RT ln (reaction quotient) , which (together with ΔG^0 = - RT ln K) simplifies to

ΔG= RT ln (reaction quotient / K)

ΔG is therefore negative (i.e. yields a spontaneous reaction) whenever the reaction quotient is lower than K. In a mitochondrion with initial pH=7.8 (and therefore pOH=6.2), the ejection of any minute amount of H^+ will therefore yield a higher pH (7.8+ε) and the ΔG for the auto-protolysis will become:

ΔG= RT ln ($[H^+][HO^-]$ / K)
ΔG= RT ln $10^{-(7.8+\varepsilon)}10^{-6.2}/ 10^{-14}$)
ΔG= RT ln $10^{-(14+\varepsilon)}/ 10^{-14}$) = RT ln $10^{-\varepsilon}$ = - εRT , which is negative and therefore tells us that auto-protolysis a spontaneous reaction under these conditions, as can also be trivially shown from an analysis of the equilibrium condition and applying Le Chatelier's principle: upon removing products from a system in equilibrium, the system WILL necessarily evolve in the direction of

generating such products. No serious user of thermodynamics can fail to know that the argument that Nath just got into print in the respected pages of “Biophysical Chemistry” is obviously wrong, and would imply (for example) that if some Ca^{2+} were removed from a saturated solution of $Ca_3(PO4)_2$ where a large chunk of solid $Ca_3(PO4)_2$ is present, no portion of the solid could ever solubilize to reach a new equilibrium (since the Kps of calcium phosphate is around 10^{-33}, ΔG^0= 188.2 kcal/mol, and therefore the reaction would be, according to Nath, even less favourable than the water auto-protolysis that he claims cannot exist), in flagrant contradiction to every single introductory chemistry text and to the experimental findings of every single living (or dead) chemist since the discovery of chemical equilibrium. Kinetic factors cannot be invoked to rule out auto-protolysis, either: from pK_w (14) , the water concentration when pure (55.5 mol dm^{-3}) and from the experimental rate of H^+ and HO^- association (1.4×10^{11} $dm^3\,mol^{-1}\,s^{-1}$), the reaction rate for water dissociation into H^+ and HO^- has been determined as 2.5×10^{-5} $mol^{-1}\,s^{-1}$ [41]. The numerical solution of these differential equations (performed with COPASI[42]) shows that when a pure water solution with an initial pH of 7.8 has lost all its H^+, enough H^+ to reach 99% of the equilibrium concentrations ($[HO^-]_{eq}=6.45 \times 10^{-7}$ mol dm^{-3} and $[H^+]_{eq}= 1.536 \times 10^{-7}$ mol dm^{-3}) can be generated in only 50 μs. In physiological conditions, where protonated buffers are present (such as amines), equilibrium is attained even more rapidly because they self-dissociate much faster than water: e.g. protonated amines release H^+ at rates between 4.1 and 24.2 s^{-1} (computed from the values in table 4 of [43])

Finally, Nath states "**Moreover, as I have shown in these sections, whatever little is there [18] is faulty, ridden with holes and flaws, and invalid, or at least insufficient.** "
As I have shown above, no such "errors" were present in my paper. On the contrary, the "demonstrations" by Nath in this paper rely on:
1) misreadings of Mitchell's reasoning regarding charge distribution
2) claims (easily disprovable by checking standard textbooks) that the current understanding of chemiosmosis entails accepting every detail of Mitchell’s proposals
3) more crucially, thermodynamic claims that neglect basic thermodynamics and Physical Chemistry knowledge.

Peer-review must find a fine balance between appropriate thoroughness and undue intransigence, and that any peer-review system stringent enough to prevent publication of every flawed paper would also be unreasonably stifling and delay progress. To this end, Editors rely on the acumen and good-will of reviewers to detect flawed arguments and, when faced with challenges to the reigning paradigms, may understandably err on the side of allowing unorthodox views to appear over

reviewers' objections to avoid unduly favoring the *status quo*. Fair-minded editors (correctly) do not want to clip the wings of the next Pegasus. Icarus, however, would have been better served if his wings had been taken from him before he had relied on their flimsiness to fly too close to the Sun.

Acknowledgments: This work was supported by the Applied Molecular Biosciences Unit - UCIBIO which is financed by national funds from FCT (UIDB/04378/2020).

References:

[1] S. Nath, Molecular-level understanding of biological energy coupling and transduction: Response to "Chemiosmotic misunderstandings," Biophys. Chem. (2020) 106496. https://doi.org/10.1016/j.bpc.2020.106496.

[2] P.J. Silva, Chemiosmotic misunderstandings, Biophys. Chem. 264 (2020) 106424. https://doi.org/10.1016/j.bpc.2020.106424.

[3] M. Morange, What history tells us XI. The complex history of the chemiosmotic theory, J. Biosci. 32 (2007) 1245–1250. https://doi.org/10.1007/s12038-007-0133-x.

[4] J. Prebble, B. Weber, Wandering in the gardens of the mind: Peter Mitchell and the making of Glynn, 2003.

[5] J.N. Prebble, Successful theory development in biology: A consideration of the theories of oxidative phosphorylation proposed by Davies and Krebs, Williams and Mitchell, Biosci. Rep. 16 (1996) 207–215. https://doi.org/10.1007/BF01207335.

[6] J.N. Prebble, The philosophical origins of Mitchell's chemiosmotic concepts: The personal factor in scientific theory formulation, J. Hist. Biol. 34 (2001) 433–460. https://doi.org/10.1023/A:1012946715673.

[7] J. Prebble, Bioenergetics and Peter Mitchell, Trends Biochem. Sci. 27 (2002) 394–395. https://doi.org/10.1016/s0968-0004(02)02156-4.

[8] J. Prebble, Peter Mitchell and the ox phos wars, Trends Biochem. Sci. 27 (2002) 209–212. https://doi.org/10.1016/S0968-0004(02)02059-5.

[9] B.H. Weber, J.N. Prebble, An issue of originality and priority: The correspondence and theories of oxidative phosphorylation of Peter Mitchell and Robert J.P. Williams, 1961-1980, J. Hist. Biol. 39 (2006) 125–163. https://doi.org/10.1007/s10739-005-3052-4.

[10] M. Weber, Theory testing in experimental biology: The chemiosmotic mechanism of ATP synthesis, Stud. Hist. Philos. Sci. Part C Stud. Hist. Philos. Biol. Biomed. Sci. 33 (2002) 29–52. https://doi.org/10.1016/S1369-8486(01)00016-4.

[11] M.D. Brand, B. Reynafarje, A.L. Lehninger, Stoichiometric relationship between energy-dependent proton ejection and electron transport in mitochondria., Proc. Natl. Acad. Sci. 73 (1976) 437–441. https://doi.org/10.1073/pnas.73.2.437.

[12] M.D. Brand, A.L. Lehninger, H+/ATP ratio during ATP hydrolysis by mitochondria: modification of the chemiosmotic theory., Proc. Natl. Acad. Sci. 74 (1977) 1955–1959. https://doi.org/10.1073/pnas.74.5.1955.

[13] M.O. Ripple, N. Kim, R. Springett, Mammalian Complex I Pumps 4 Protons per 2 Electrons at High and Physiological Proton Motive Force in Living Cells, J. Biol. Chem. 288 (2013) 5374–5380. https://doi.org/10.1074/jbc.M112.438945.

[14] A.R. Crofts, S. Lhee, S.B. Crofts, J. Cheng, S. Rose, Proton pumping in the bc1 complex: A new gating mechanism that prevents short circuits, Biochim. Biophys. Acta - Bioenerg. 1757 (2006) 1019–1034. https://doi.org/10.1016/j.bbabio.2006.02.009.

[15] G. Antonini, F. Malatesta, P. Sarti, M. Brunori, Proton pumping by cytochrome oxidase as studied by time-resolved stopped-flow spectrophotometry., Proc. Natl. Acad. Sci. 90 (1993) 5949–5953. https://doi.org/10.1073/pnas.90.13.5949.

[16] D. Williamson, P. Lund, H. Krebs, The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver, Biochem. J. 103 (1967) 514–527. https://doi.org/10.1042/bj1030514.

[17] E.G. Mik, T. Johannes, C.J. Zuurbier, A. Heinen, J.H.P.M. Houben-Weerts, G.M. Balestra, J. Stap, J.F. Beek, C. Ince, In Vivo Mitochondrial Oxygen Tension Measured by a Delayed Fluorescence Lifetime Technique, Biophys. J. 95 (2008) 3977–3990. https://doi.org/10.1529/biophysj.107.126094.

[18] J. Dzbek, B. Korzeniewski, Control over the contribution of the mitochondrial membrane potential (ΔΨ) and proton gradient (ΔpH) to the protonmotive force (Δp): In silico studies, J. Biol. Chem. 283 (2008) 33232–33239. https://doi.org/10.1074/jbc.M802404200.

[19] R.S. Cockrell, E.J. Harris, B.C. Pressman, Energetics of Potassium Transport in Mitochondria Induced by Valinomycin *, Biochemistry. 5 (1966) 2326–2335. https://doi.org/10.1021/bi00871a022.

[20] E.C. Slater, An Evaluation of the Mitchell Hypothesis of Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Eur. J. Biochem. 1 (1967) 317–326. https://doi.org/10.1111/j.1432-1033.1967.tb00076.x.

[21] E.C. Slater, Peter Dennis Mitchell, 29 September 1920 - 10 April 1992, Biogr. Mem. Fellows R. Soc. 40 (1994) 283–305. https://doi.org/10.1098/rsbm.1994.0040.

[22] E.C. Slater, Metabolic gardening, Nature. 422 (2003) 816–817. https://doi.org/10.1038/422816a.

[23] E.C. Slater, The mechanism of the conservation of energy of biological oxidations, Eur. J. Biochem. 166 (1987) 489–504. https://doi.org/10.1111/j.1432-1033.1987.tb13542.x.

[24] R.J.P. Williams, Some unrealistic assumptions in the theory of chemi-osmosis and their consequences, FEBS Lett. 102 (1979) 126–132. https://doi.org/10.1016/0014-5793(79)80943-6.

[25] R.J.P. Williams, The history of proton-driven ATP formation, Biosci. Rep. 13 (1993) 191–212. https://doi.org/10.1007/BF01123502.

[26] B. Williams, Bioenergetics and Peter Mitchell, Trends Biochem. Sci. 27 (2002) 393–394. https://doi.org/10.1016/s0968-0004(02)02138-2.

[27] R.J.P. Williams, Chemical advances in evolution by and changes in use of space during time, J. Theor. Biol. 268 (2011) 146–159. https://doi.org/10.1016/j.jtbi.2010.09.021.

[28] R. Chang, Physical Chemistry for the Chemical and Biological Sciences, 3rd Editio, University Science Books, 2000.

[29] P. Mitchell, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, Glynn Research Laboratories, Bodmin, United Kingdom, 1966.

[30] P. Mitchell, Chemiosmotic coupling and energy transduction, Glynn Research Laboratories, Bodmin, United Kingdom, 1968.

[31] D.G. Nicholls, S.J. Ferguson, Bioenergetics 3rd Edition, Full Text via CrossRef| View Rec. Scopus| Cited By Scopus. (2002) 1–333.

[32] S. Nath, Modern theory of energy coupling and ATP synthesis. Violation of Gauss's law by the chemiosmotic theory and validation of the two-ion theory, Biophys. Chem. 255 (2019) 106271. https://doi.org/10.1016/j.bpc.2019.106271.

[33] P. Mitchell, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, Biochim. Biophys. Acta - Bioenerg. 1807 (2011) 1507–1538. https://doi.org/10.1016/j.bbabio.2011.09.018.

[34] D.H. Song, J. Park, L.L. Maurer, W. Lu, M.A. Philbert, A.M. Sastry, Biophysical significance of the inner mitochondrial membrane structure on the electrochemical potential of mitochondria, Phys. Rev. E. 88 (2013) 062723. https://doi.org/10.1103/PhysRevE.88.062723.

[35] R.P. Feynman, R.B. Leighton, M. Sands, The Feynman Lectures on Physics. Volume II: Mainly electromagnetism and matter, 1966.

[36] H. Guo, G.M. Courbon, S.A. Bueler, J. Mai, J. Liu, J.L. Rubinstein, Structure of mycobacterial ATP synthase with the TB drug bedaquiline, BioRXiv. (2020). https://doi.org/10.1101/2020.08.06.225375.

[37] D. Edgington, Indicative Conditionals, Stanford Encycl. Philos. Edward N. Zalta. (2020). https://plato.stanford.edu/entries/conditionals/ (accessed November 2, 2020).

[38] M. Ohshima, S. Ohno, S. Nakajin, Inhibitory effects of some possible endocrine-disrupting chemicals on the isozymes of human 11beta-hydroxysteroid dehydrogenase and expression of their mRNA in gonads and adrenal glands., Environ. Sci. 12 (2005) 219–30. http://www.ncbi.nlm.nih.gov/pubmed/16184081.

[39] S. Harada, Y. Hiromori, S. Nakamura, K. Kawahara, S. Fukakusa, T. Maruno, M. Noda, S. Uchiyama, K. Fukui, J. Nishikawa, H. Nagase, Y. Kobayashi, T. Yoshida, T. Ohkubo, T. Nakanishi, Structural basis for PPARγ transactivation by endocrine-disrupting organotin compounds, Sci. Rep. 5 (2015) 8520. https://doi.org/10.1038/srep08520.

[40] A. le Maire, M. Grimaldi, D. Roecklin, S. Dagnino, V. Vivat-Hannah, P. Balaguer, W. Bourguet, Activation of RXR–PPAR heterodimers by organotin environmental endocrine disruptors, EMBO Rep. 10 (2009) 367–373. https://doi.org/10.1038/embor.2009.8.

[41] F.H. Stillinger, Proton transfer Reactions and Kinetics in Water, in: Theor. Chem. Adv. Perspect., Academic Press, New York, 1976: pp. 178–234.

[42] P. Mendes, S. Hoops, S. Sahle, R. Gauges, J. Dada, U. Kummer, Computational modeling of biochemical networks using COPASI., Methods Mol. Biol. 500 (2009) 17–59. https://doi.org/10.1007/978-1-59745-525-1_2.

[43] M. Eigen, Proton Transfer, Acid-Base Catalysis, and Enzymatic Hydrolysis. Part I: ELEMENTARY PROCESSES, Angew. Chemie Int. Ed. English. 3 (1964) 1–19. https://doi.org/10.1002/anie.196400011.

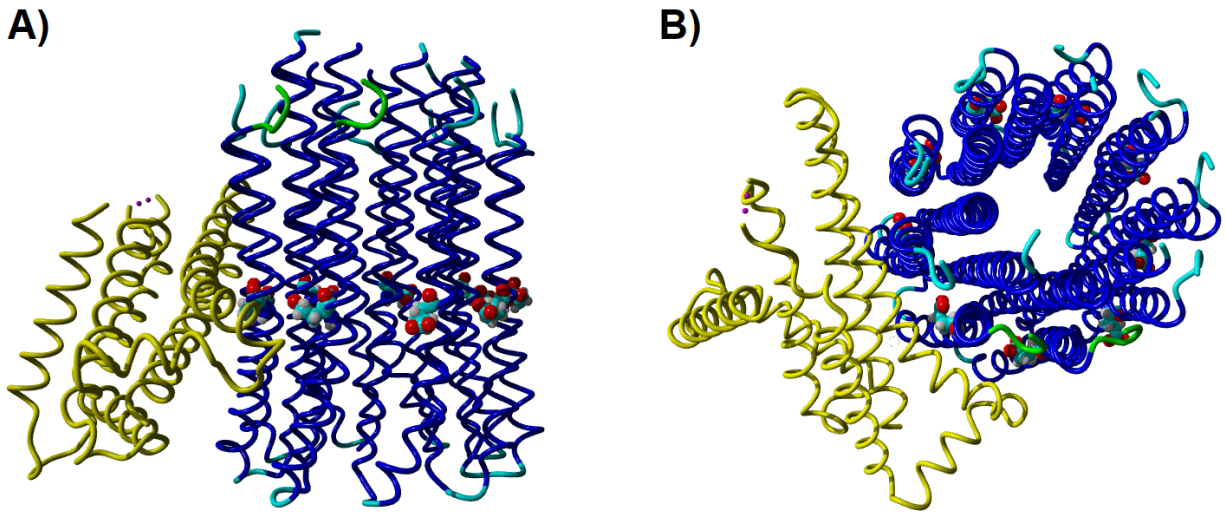

Figure 1: The interface between ATP synthase a subunit and the c-ring (PDB:7JG5)[36]. A) “side view”. B) “Top” view. The atoms of the Glu65 residues of the c-subuints (which are widely accepted to receive the protons from one half-channel in the a-subunit and feeding them to the other half-channel) are shown as solid spheres.

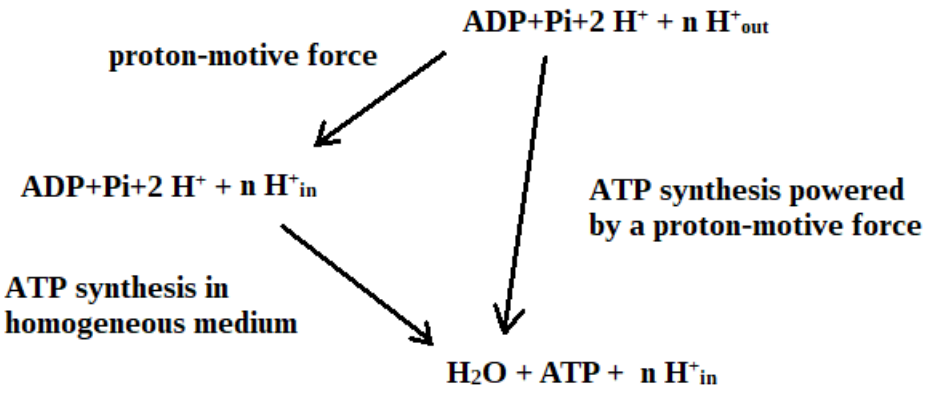

Figure 2: Thermochemical cycle showing the computation of the proton motive-force from the comparison of the ΔG of ATP synthesis in the coupled reaction with the un-assisted reaction ("ATP synthesis in homogeneous medium").