Chemiosmosis principle versus murburn concept: *Why do cells need oxygen?* Deducing the underpinnings of aerobic respiration by mechanistic predictability

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Abstract: The long-standing explanation for cellular respiration (mitochondrial oxidative phosphorylation, mOxPhos) in textbooks is proton-centric and involves the elements of Rotary ATP synthesis, Chemiosmosis principle, Proton pumps and Electron transport chain (in short, the RCPE model). Addressing certain lacunae in the RCPE model, an alternative scheme based on murburn concept was proposed in 2017 (Manoj, 2017). The new proposal is oxygen-centric in essence, and it advocates constructive roles for diffusible reactive oxygen species (DROS) in electron transfer reactions and ATP-synthesis. By the end of 2018, significant arguments and experimental evidences (*in vitro*, *in situ*, and *in silico*) had accumulated supporting the new mechanism. Herein, the authors compare the predictive capabilities of the two models. Theoretical concepts and expectations are detailed to differentiate the two models, and the correlations are cross-checked with the available data/information. Experimental strategies are further charted to delineate and demarcate the two hypotheses' relevance in mOxPhos.

Keywords: cellular/aerobic respiration, mitochondria, ATP synthesis, respiratory complexes, oxidative phosphorylation, murburn concept, chemiosmosis, electron transport chain (ETC),

The heads and tails of the RCPE and murburn models for oxidative phosphorylation

The chemiosmosis explanation for cellular respiration was proposed by Peter Mitchell (Mitchell, 1961). He postulated that the protein complexes embedded in the inner mitochondrial membrane pump protons from matrix into the inter-membrane space (IMS). This proton-surplus in the IMS yields a trans-membrane chemical/proton differential or potential (TMP), which drives ATP synthesis in the mitochondrial matrix. When this hypothesis was proposed, not even a shred of evidence existed to support the idea (Prebble, 2001; Prebble and Weber, 2003). However, within a couple of decades, several researchers went on to support this paradigm (Jagendorf and Uribe, 1966; Reid, Moyle and Mitchell, 1966; Junge, Schliephake and Witt, 1968; Liberman, Topaly and Tsofin, 1969; Racker and Stoeckenius, 1974). Particularly in that regard, Paul Boyer hypothesized that the influx of IMS protons into the matrix (through the proton pore of Complex V) serves as an electro-mechanical link between electron transfers (ET) and phosphorylation. Therefore, Complex V is understood to be a rotary ATP synthase (Boyer, 1997). This proton-centric model shall be henceforth referred to as the RCPE model and its salient components/features are depicted in Figure 1. In the Mitchell-Boyer paradigm, diffusible reactive oxygen species (DROS) are unaccounted and considered as wasteful/toxic products. The RCPE working machine model can be compared with a hydroelectric plant (water-mill) or an automobile. The proton-centric scheme fell short to explain the theoretical higher ATP yield mandated by cell metabolic needs and the experimental reports of higher efficiency by several researchers (Lee *et al.*, 1996; Hinkle, 2005). In spite of the recognition accorded to the RCPE model, several leading researchers continued to question the same owing to its inherent incongruities (Williams, 1979; Ling, 1981; Wainio, 1985; Nałęcz, 1986; Slater, 1987; Nath, 2010). Moreover, the fact that mitochondria are practically proton-limited microcosms proved to be this model's Achilles' heel (Manoj, 2017, 2018a).

To overcome such limitations of the chemiosmosis hypothesis, Kelath Murali Manoj (KMM) proposed an evidence-based oxygen-centric explanation for mOxPhos in 2017 (Manoj, 2017). This new understanding was built on experimental insights gained from- (i) KMM's group's works on heme and flavin protein systems (Manoj and Hager, 2001, 2008; Manoj *et al.*, 2010; Manoj, Gade and Mathew, 2010; Andrew, Hager and Manoj, 2011; Gade, Bhattacharya and Manoj, 2012; Parashar and Manoj, 2012; Gideon *et al.*, 2012; Parashar, Gade, *et al.*, 2014;

Parashar, Venkatachalam, *et al.*, 2014; Manoj, Parashar and Venkatachalam, 2015; Manoj, Venkatachalam and Parashar, 2016; Venkatachalam, Parashar and Manoj, 2016; K.M. Manoj, Parashar, Gade, *et al.*, 2016; K.M. Manoj, Parashar, Venkatachalam, *et al.*, 2016; Kelath Murali Manoj *et al.*, 2016), (ii) newly available structural details of mitochondrial protein complexes (Fiedorczuk *et al.*, 2016; Wu *et al.*, 2016), and (iii) accumulated awareness on cell/mitochondrial physiology (Picard *et al.*, 2011; Zorov, Juhaszova and Sollott, 2014; Sazanov, 2015). For the mOxPhos reaction system, this mechanistic view considers membrane complexes as ADP-binding and oxygen-activating (DROS generating and modulating) agents and protons as a “crucial limiting reactant” (affecting several equilibria). In the murburn model, DROS serve as the “chemical coupling agent” which directly links NADH oxidation with ATP synthesis (Manoj *et al.*, 2019). Herein, trans-membrane potential (TMP) formation is seen as a consequence of high amounts of negatively charged transient oxygen-centered species formed within the matrix, around the inner mitochondrial membrane. This oxygen-centric model shall be henceforth referred to as murburn model and its elements are shown in Figure 2. A grill/hearth or nuclear reactor is comparable with the murburn model of mOxPhos.

Figure 1: The RCPE hypothesis has two independent ‘deterministic’ parts that solicit a staggered operational/regulatory mechanistic sequence. First, a proton differential (set up between the matrix and inter-membrane space, IMS) needs to be generated by the ETC (which also serve as proton pumps) and then the chemiosmotic proton motive force (pmf) or/and trans-membrane potential (TMP) are harvested to synthesize ATP through a rotary function of Complex V. Oxygen is needed/reacted only at Comp. IV.

Figure 2: The Murburn scheme involves stochastic interactions and equilibriums, and obligatorily requires oxygen/DROS at several points. DROS is needed as activating or catalytic agents and the whole system works in steady-state, with Complex V serving as a trans-membrane conduit for cytoplasmic protons. The activation of ADP/Pi for the synthesis of ATP occurs at multiple loci.

Table 1 delineates the roles of key components of the two models and the fundamental mechanistic differences between them.

Table 1: Salient mechanistic differences between the physiological models for mOxPhos.

No.	Facets / Roles	RCPE model	Murburn model
1	Protein Complexes (Protons & proton pumps)	Membrane-proteins network to form an ETC and also serve as proton pumps. They employ copious amounts of protons as key operative agents.	Membrane complexes are not proton pumps but ADP-binders cum DROS generators (modulators). Protons influence multiple reaction equilibriums and kinetics thereof.
2	Oxygen/DROS	Oxygen is the terminal electron acceptor and DROS are toxic/waste products.	Oxygen is the key operative agent required for forming the catalytically important DROS.
3	TMP (pmf)	Pivotal driving force that recycles matrix protons and synthesizes ATP.	Secondary result that aids the inward movement of cytoplasmic protons/cations.
4	ATP-synthesis in steady-state	Complex V is the sole agent, which requires only IMS protons.	All membrane complexes and cytoplasmic protons are involved.
5	Connectivity between ET and ATP-synthesis	No direct connection; indirect electro-mechanical connectivity via protons.	DROS directly connects ET with ATP-synthesis via a catalytic reaction scheme.

A critical appraisal of the fundamental predictive abilities of the two models

The mandate of this manuscript is to compare the two models for the predictability of outcomes with respect to the experimental data currently available on the mOxPhos system. With reference to the context of the major mechanistic criteria highlighted in Table 1, a critical discussion is presented in a tabular form within Table 2. Furthermore, the citations to the information listed therein can be available from KMM's recent published works (Manoj, 2018a, 2018b; Manoj, Gideon and Jacob, 2018; Manoj *et al.*, 2019). Other information perused herein are supported by well-known biochemistry textbooks like Lehninger, Stryer and Voet (Berg, Tymoczko and Stryer, 2002; Lehninger, Nelson and Cox, 2004; Voet and Voet, 2011).

Table 2: Predictive accuracy of the fundamental tenets of the two models.

No.	Predictive Query	Relevant Facts	Direct deduction
1.	<i>If the ETC proteins pump protons, mitochondria must have ample free protons. Further, the reaction at the ETC Complexes should afford enough energy to pump the required amounts of protons.</i>	While several tens of thousands of ETC proteins are present in a mitochondrion, protons are sparse (at physiological pH). Only the reaction at Comp. IV affords the thermodynamic "window" to pump two protons per passage of electron.	The murburn model does not invoke proton pumps and deems protons as a limiting reactant. Thus, this model can operate in the practically aprotic mitochondrial environment.
2.	<i>If the ETC proteins served as ADP-binders and DROS generators and/or modulators, one would expect ADP-binding sites and DROS access channels/cavities within them.</i>	Earlier <i>in vitro</i> experimental works have indicated the presence of ADP-binding sites on ETC Complexes. The recent structural and <i>in silico</i> works have pointed out the loci of the ADP-sites and the O ₂ -accessible redox centers.	The recent findings support the murburn model. The presence of O ₂ -accessible redox centers in proteins discount the RCPE scheme. Since proton-pumps are not tenable, ETC complexes serve no role other than water formation.
3.	<i>If oxygen served only as the terminal electron acceptor and stayed bound to Comp. IV alone, other ETC complexes should not generate DROS.</i>	The highly mobile oxygen being restricted to Comp. IV alone has no chemical or physical basis. Ample experimental evidence show that that Complexes I through IV generate and modulate DROS.	The deterministic nature of RCPE model does not correspond to the facts. The entry and outward leak of protons at multiple points supports the murburn model.

4.	<i>If DROS is the key protagonist in mOxPhos, DROS formation would increase with higher amounts of NADH/O₂ in mitochondria..</i>	Higher amounts of NADH and O ₂ generate greater amounts of DROS in mitochondrial physiology.	While the RCPE model does not address mitochondrial propensity for DROS formation, murburn scheme explains the mobility and reactivity of oxygen, and DROS formation.
5.	<i>If a proton-based TMP is generated and sustained across the inner mitochondrial membrane (between the matrix and IMS), then the outer mitochondrial membrane should be impermeable to protons (so that escape of protons via the same is prevented).</i>	The pH of IMS in mitochondria and periplasm in prokaryotes equilibrates with the pH of cytoplasm and external medium respectively. The TMP has been demonstrated only between matrix and cytoplasm.	The reality/facts discount the steady state tenability of the RCPE model. Murburn model states that a TMP (across the matrix and cytoplasm, not IMS) arises due to the generation of negatively charged DROS in the matrix, not by a proton differential.
6.	<i>If the TMP enables Comp. V to change conformation and binding affinities, it must have a TMP sensor, enabling it to temporally modulate and shift between its affinities for ADP and ATP.</i>	The structures of several ATPases are known now. There are no components to sense or tap any electro-static/magnetic fields within Complex V. A fluctuating potential is not observed. (Also, the proton numbers within a mitochondrion cannot account for the rate of ATP-synthesis.)	The TMP or proton-based explanation cannot account for physiological ATP synthesis. This scenario is incompatible with RCPE model. DROS catalyzes the ATP synthesis without a proton gradient or TMP, which supports the Murburn Scheme.
7.	<i>If Comp. V is the physiological ATP-synthase, it would have higher affinity for ADP, when compared to ATP.</i>	Comp. V has more than ten million folds higher affinity for ATP (compared to ADP). So, if Comp. V is an ATP synthesizer, the mitochondrial ATP levels must be lower than ADP by several orders.	The thermodynamic and kinetic realities preclude the RCPE model. However, it is unclear how ADP or ATP binding to Comp. V affects the murburn model.
8.	<i>Can we get higher rate of ATP-synthesis with increased NADH+O₂? Vice versa, will the addition of ADP+Pi increase NADH+O₂ consumption? In the RCPE model, ET rate, a limiting criterion, is independent of ADP+Pi.</i>	We get higher rate in both of the physiological scenarios. The limiting factors of mOxPhos are proton availability and CoQ mobility, which are independent of NADH+O ₂ or ADP+Pi concentrations and/or consumption.	Since the ET and ATP-synthesis are disconnected in RCPE model, it cannot explain the increase in metabolic rate under the two scenarios. The murburn model posits that DROS production causes a thermodynamic pull that catalyzes the ATP synthesis.

9.	<i>If DROS generates TMP and catalyzes ATP synthesis (murburn model), one would predict a direct correlation of DROS with TMP and ATP synthesis in a respiring mitochondrion.</i>	In respiring mitochondria, TMP generation and ATP synthesis directly correlates with DROS production.	RCPE cannot explain this outcome, whereas this result is consistent with the murburn model.
10.	<i>If DROS serves to esterify ADP with Pi in mitochondrial physiology (murburn model), can we simulate the reaction in vitro?</i>	Experimental evidence shows that in mitochondria and in vitro setups, superoxide esterifies inorganic phosphate to ADP (yielding ATP).	Such observations downplay the RCPE hypothesis (which deems DROS as a mere toxic waste product) and conclusively support the murburn scheme.

From the above, it can be seen that RCPE model fails to shoulder the fundamental mechanistic tenets it proposes. At the outset, murburn concept appears to be a more facile chemical logic that is neither self-contradictory nor does it seek insurmountable chemico-physical requisites.

Murburn versus Chemiosmosis: Miscellaneous predictability of the two models

Essentially, ATP synthesis is a chemical reaction occurring in space and time. So, the fundamental elements of this reaction are- the nature of the reaction vessel, the catalysts involved, and the reaction propensities of the reactants. Further, biochemistry relies on elucidating the reaction mechanism through incorporation of additives (inhibitors/modulators). So, the following discussion focuses on these areas as the criteria for the mechanistic differentiation of the two models. (The points are numbered starting from 11, to continue with the agenda of comparison started in Table 2.)

Structure and distribution of ETC complexes in Mitochondria

11. *As per the RCPE model, a tightly packed system of protein complexes and a greater ratio of Comp. I/II (flavoprotein):Comp. IV (hemoprotein) is essential for an efficient ETC and electron transfer stoichiometry, respectively.*

The distribution of respiratory proteins around the mitochondrial inner membrane is shown in Figure 3. The mean distance between the two nearest proteins/complexes ranges between 7 – 20

nm, and they are distributed at a density of 10^{10} to 10^{11} molecules per cm^2 (occupying ~50% surface area of the membrane). The range of protein complexes are $\sim 10^4$ - 10^5 per mitochondrion (Gupte *et al.*, 1984; Schwerzmann *et al.*, 1986). This means that RCPE model mandates that “batteries” (serially networked protein complexes) of $\sim 10^4$ - 10^5 ETC complexes exist per mitochondrion. The concentration of Comp. IV (DROS modulator) is significantly higher than Comp. I/II (primary ROS generators) (Schägger and Pfeiffer, 2001). Thus, the relative distribution densities of protein complexes and components support the murburn model. For the RCPE model to operate, the concentration of Complex I/II must be higher than that of Complex IV. Furthermore, the ratio of Comp. IV to Cyt. *c* (1:1.5) is disadvantageous for the RCPE model. The murburn scheme is a parallel system (unlike the serial arrangement of components in the RCPE model) and hence, the relative distribution densities of protein complexes and reaction rates are better explained by the murburn model (Manoj *et al.*, 2019).

Figure 3: General architecture of a mitochondrion (A) and an analysis of the distribution ratios of key proteins (B). The RCPE model posits a deterministic or fixed ratio of these protein complexes (2:2:4:1), while the actual distribution is quite the opposite. The murburn model supports the observed distribution of ETC (see text for details).

12. Since several electron transfer steps are rate limiting, one would imagine that the sequential ETC would get optimized through evolution to- (i) obviate connectivity issues, and (ii) minimize DROS formation. Also, for the proton gradient to form, there must be a modularized and synchronized network of the proton-pumping ETC-complexes.

The features of- (i) non-sophisticated architecture of mitochondria, (ii) random distribution of protein complexes in the membrane, (iii) requirement 2 CoQ and 4 Cyt. *c* molecules to ferry $4e^-$

for the reduction of one O₂ molecule, (iv) the presence of non-favorable potential gradients and distances between redox centers, (v) non-reducible and non-route redox centers, (vi) requirement of protons within membrane embedded loci, etc. counter the sequential/serial and deterministic RCPE model. Mitochondria do not have nano-compartmentalization to generate multiple localized regions with fluctuating pH. Further, it is not clear how the protein complexes could function serially, in the form a “battery”, all the while avoiding DROS formation. Therefore, the reality seems to be a deliberate ploy by life’s evolutionary principles to enable “a closed pot reaction” logic for achieving DROS-mediated ETs/catalysis. The structural aspects listed above support the murburn model and discount the RCPE model.

13. If Comp. I/II collect electrons (from NADH and succinate respectively) and pass it to the membrane-based CoQ, they would have a short matrix-arm (with occluded redox centers) and long trans-membrane foot with well-separated redox centers (to enable proton pumping).

A systematic comparison of minimal requirement of the structural attributes by the two models is shown in Figure 4. Both Comp. I & II have a large bulbous matrix-ward extension with multiple redox centers. There is little structural attribute in the trans-membrane foot that suggests a proton pumping functionality. Since electron transfer rates are several orders faster than the purported proton pump rates, the two processes can’t be spatio-temporally coupled. Therefore, how the RCPE model Complex I synchronizes ET and proton pumping is obscure. Murburn scheme seeks the generation of DROS by the two flavoenzymes of Comp. I and II (Manoj, Gade and Mathew, 2010; K.M. Manoj, Parashar, Gade, *et al.*, 2016; Manoj *et al.*, 2019). The presence of multiple redox centers in the matrix domains of Complex I and II aids DROS-generation (Hatefi, 1985; Grivennikova and Vinogradov, 2006; Quinlan *et al.*, 2012; Manoj *et al.*, 2019), which supports the murburn model (Figure 4 A). Especially, the RCPE model does not explain the functionality of the large matrix domain of Complex III (Figure 4B), which could well operate with just the trans-membrane domain of the Complex. The fact that the matrix domain of this complex houses ADP binding sites and cavities (enabling DROS-dynamics) (Manoj *et al.*, 2019) supports the murburn model. In short, the matrix domains of Complexes I-IV house DROS-generating/modulating redox centers and ADP binding sites, which are essential requisites of the murburn scheme.

Figure 4: Comparison of structural attributes of the redox protein complexes (Complexes I to IV) of mitochondrial aerobic respiration (A-D). RCPE mode deems the matrix domain of these complexes redundant. However, the presence of multiple redox centers and ADP binding sites in these domains supports the Murburn model. Only a single monomer's redox centers are depicted in Comp. IV (D).

14. *The RCPE model requires a mitochondrial membrane impermeable to protons, whereas the murburn model doesn't. Also, the RPCE model predicts/seeks the mitochondrial membranes to be highly restrictive to protons whereas, the murburn model doesn't. It is difficult to envisage that a non-specific inward proton flux (via the lipid membrane) can be prevented in a functional mitochondrion.*

The overall equation of mOxPhos starting from NADH is proton deficient and the ETC concept clearly vouches for the separation of protons from electrons. Moreover, only close-packed membrane proteins could potentially restrict the proton influx. Protons generically permeate the phospholipid membrane to small levels, in millisecond time ranges (Biegel and Gould, 1981). As per the murburn model, reduced CoQ takes up such generic proton influx. Accounting for proton availability and dynamics is the biggest handicap of the proton-centric RCPE model.

15. *As per the murburn model, how does the mitochondrial membrane adapt to the formation and stabilization of DROS in mOxPhos?*

The preponderance of cardiolipin, a special phospholipid, affords high negative charge densities at the membrane interface, which retains negatively charged species within the matrix. Since the RCPE model seeks both inward and outward proton flow, it cannot explain the presence of cardiolipin and evolutionary conservation of this lipid in mitochondria. The distribution of cardiolipin is conserved across the mitochondrial and prokaryotic membranes alike, which suggests its DROS-restrictive significance in the ubiquitous mOxPhos physiology. CoQ can also scavenge DROS that otherwise manages to collide with the membrane.

Different aspects of the reaction chemistry and the effect of additives

16. *Since the deterministic RCPE model has definitive distribution of ETC and defined $2e^-$ reaction equations, it should have well-defined stoichiometries (P/O and H/P) for both succinate and NADH.*

Studies across labs show variable and non-integral stoichiometries (H:P or O:P) in mOxPhos (Hinkle, 2005; Watt *et al.*, 2010) and this does not support the deterministic $2e^-$ scheme of RCPE, which also advocates proton pumps to work at batches of 4 or 2. The lack of a well-

defined stoichiometry (and thereby the difficulty in arriving at precision or accuracy) is the classical signature of a stochastic murburn scheme.

17. *The RCPE model predicts a constant (high) loss of redox equivalents at all times, in the presence or absence of ADP+Pi.*

The depletion rate of NADH is higher and lower respectively in the presence and absence of ADP+Pi (Manoj, 2017; Manoj *et al.*, 2019). The redox-pull mechanism of murburn model explains this observation, which is quite analogous to the microsomal xenobiotic metabolism (K.M. Manoj, Parashar, Gade, *et al.*, 2016).

18. *Any disruption in mitochondrial membrane affects the RCPE model as it requires an ordered arrangement of protein complexes.*

Membrane disruption does not significantly affect the depletion of NADH and consumption of oxygen (Berg, Tymoczko and Stryer, 2002; Lehninger, Nelson and Cox, 2004; Voet and Voet, 2011). The RCPE model requires ordered arrangement protein complexes and movement of its essential components, which means that any deviation from this deterministic model critically affect the depletion of NADH and consumption of oxygen.

19. *According to classical kinetics (RCPE model), the K_d of oxygen binding (equilibrium dissociation constant) with Complex IV should be lower than K_M (Michaelis constant).*

Quite unusually, $K_d \gg K_M$ for oxygen's interaction with Comp. IV (Orii, 1988; Manoj *et al.*, 2019). This maverick fact supports the murburn model of mOxPhos and negates the RCPE model.

20. *If Marcus electron transfer theory were relevant for all steps, then anoxic ET between each or every two redox centers in the route should at least be in the same order as the overall ETC rate.*

The anoxic ET between the two heme *a* of Comp. IV afforded several orders lower rates when compared to the overall ETC rates (Orii, 1988). This finding counters the deterministic ET scheme of RCPE. The ET paradigm could be explained by murburn model.

21. *If the electron transport works as per the murburn model, we would expect maverick concentration- and dose-dependent effects of various amphipathic toxins and uncouplers such as disubstituted phenolics.*

Maverick concentration-dependent effects were reported in biological phosphorylations by various groups in the 1960s to 1970s (Avron and Shavit, 1965; Watling-Payne and Selwyn, 1974), only to be side-tacked as artifacts. A dose-dependent effect of amphipathic respiratory toxins and uncouplers of electron transport chain has been reported before (Kovacic *et al.*, 2005; Manoj, 2018b). Disubstituted phenolics are uncouplers in xenobiotic metabolism mediated in microsomes too (Tokutake, Miyoshi and Fujita, 1991; Parashar, Gade, *et al.*, 2014). These observations support the operational relevance of murburn concept. Dynamic DROS modulation at the phospholipid interface could explain such effects.

22. *If cyanide inhibited mOxPhos by the RCPE model (via binding to the heme center of Complex IV), then the toxicity levels for cyanide administration would approach g/Kg doses (because the effective K_d of cyanide would be in high millimolar ranges).*

The oral/blood toxicity of cyanide falls in mg/Kg ranges discounts the relevance of RCPE mechanism (Manoj *et al.*, 2019). Since cyanide is not supposed to bind with Complexes I, II, III, and V (Way, 1984), the first three proton pumps should enable ATP production via the influx of protons through Complex V, as the RCPE model suggests. Oxygen accepts electrons from Complexes I-III too, and if the RCPE model is valid, the proton pumps should theoretically work and the Complex V should synthesize ATP. But aerated and NADH replenished mitochondrial systems do not show ATP formation in the presence of cyanide (Aw and Jones, 1989). The low lethal dose of cyanide implicates the diffusible role of cyanide (Parashar, Venkatachalam, *et al.*, 2014; Manoj *et al.*, 2019) in DROS mediated ATP synthesis and thereby ratifies the relevance of murburn model. Thus, the findings also negate the RCPE hypothesis.

23. *If murburn model explains the phosphorylation chemistry, we could also observe non-specific phosphorylations in biological systems with DROS-generating agents.*

ROS-associated non-specific phosphorylation of hydroxyl groups of amino acids and xenobiotics have been noted in literature (Spudich and Stoeckenius, 1980; Stadtman and Levine, 2003; Uchida, 2003; Mitchell, 2016), which reinforces the operational relevance of murburn model.

24. If a diffusible reactive species were involved in oxidative phosphorylation, one would expect to get several labeled oxygen atom's incorporation in ATP. If DROS chemistry were involved in the uncoupling phenomena, we could expect to see uncouplers inhibiting phosphorylations in other reaction systems too (which do not invoke the relevance of TMP/proton pumps).

Such observations have been reported in the field by several researchers from the 1950s to 1970s, particularly pioneered by Mildred Cohn (Cohn, 1953). These observations are one of the strongest mechanistic evidences of murburn concept's relevance in mOxPhos.

25. If murburn model were to be relevant, a DROS generator and an ATPase can synthesize ATP in a closed vesicular system.

The famed Racker's experiment (Racker and Stoeckenius, 1974) and the recent Otrin's demonstration of reconstituting ATP synthesis activity in vesicles with only oxidase and Complex V (Otrin *et al.*, 2017) support the murburn model.

26. If murburn scheme were operational, one would expect shorter life spans for animals with high metabolic rate.

This is in fact the statistical case in nature (Olshansky and Rattan, 2009) and therefore, the physiological and evolutionary relevance of murburn model is ratified.

27. The operating logic of the oxygen-centric concept invokes a hearth or nuclear reactor type of working principle.

The logic of RCPE model of mOxPhos is analogous to an automobile or hydroelectric power plant. However, a spontaneously formed automobile (with synchronized functions of engine, battery, dynamo, sensors/controllers, etc.) or hydroelectric power plant (employing the gambits of pumping something upstream first and then harvesting its flow in a purported flow downstream) does not exist. While the hearth or grill is a simple comparison, the nuclear reactor is a sophisticated analogy for the murburn mOxPhos system. There are countless examples of the

natural/spontaneous formation of a hearth or grill. The rare example of the spontaneous formation of a natural nuclear reactor (Oklo, Gabon) also lends support for the machine logic of murburn explanation of mOxPhos (Manoj, 2018b).

28. When life evolved in the primordial times, highly specific affinity-based reactions based on topological and electrostatic complementation could not have existed.

The fact that the biological ETC can take in and give out electrons through several man-made molecular/radical species (Hauska, 1977) vouches for a non-specific one-electron reaction paradigm. Such observations strengthen the relevance of murburn model in mOxPhos.

29. The overall yield of ATP production from the complete oxidation of glucose to CO₂ is about 40%. Therefore, since mOxPhos is in the last step of the overall metabolic cascade (after glycolysis and Krebs cycle), the yield of mOxPhos should be much higher than 40%.

Theoretically, the yield of mOxPhos is only 40%. Therefore, it cannot explain the higher efficiencies required and experimentally reported by several workers (Lee *et al.*, 1996). Through augmentation of metabolic control and optimization of diffusible radical species, the high efficiency of mOxPhos is achieved.

30. The respiratory ATP synthesis in anaerobic micro-organisms does not involve oxygen or DROS. This could implicate that murburn concept is not an evolvable or conserved mechanism.

Murburn scheme is essentially a molecule-ion-radical interactive scheme. In anaerobic prokaryotes, other components (sulfur and nitrogen centered species, for example) could suit the roles played by oxygen-centered protagonists in eukaryotic mitochondria. However, the mobility and discharge of the reactants and end-products (larger and precipitating species) would be a slower process, thereby explaining the slow metabolic rate of anaerobic systems.

Further experimentations proposed to demarcate the two models

In the near future, experiments detailed below could potentially ratify the relevance of murburn concept in mOxPhos and differentiate the two models.

* The arguments professed herein can be further confirmed by employing- (a) chemical controls and reductionist approach, within controlled-water systems like normal/reverse micelles, with DROS generating/stabilizing systems involving proteins containing Mg / Fe / peroxide / NADH / superoxide / pyrrhione-photolysis (as a hydroxyl radical source) and substrate analogs. (b) proteins can be engineered to incorporate a flavin and a heme cofactor with surface ADP-binding sites, in conjunction with a proton-delivering system, to demonstrate ATP synthesis in closed vesicles. (Details for protocols are provided in KMM's original arxiv preprint available at- <https://arxiv.org/ftp/arxiv/papers/1703/1703.05827.pdf>)

* Amphipathic DROS modulating molecules (like vitamin E and fatty acyl vitamin C) and trans-membrane helix containing redox-active enzymes (like horseradish peroxidase) inhibit membrane-embedded electron transport and redox metabolism in microsomal xenobiotic metabolic (mXM) system (Parashar, Gade, *et al.*, 2014); whereas their soluble functional analogs (trolox, ascorbate, and superoxide dismutase) don't inhibit (K.M. Manoj, Parashar, Venkatachalam, *et al.*, 2016) the same. Further, many of these inhibitions exhibit a maverick concentration-dependent profile (mM to pM). Such effects of various uncoupling molecules can be further probed and demonstrated in the mOxPhos system. A similar set of reactions could be repeated in a minimalistic model like the Racker's/Otrin's experiment. In conjunction, the inhibitory capacity of retinol, retinal and retinoic acids and their esters could also be compared.

* Evolutionary-lineage analysis of respiratory complexes may give evidence of DROS modulation machinery's progression and specific adaptation across domains in various species.

* The new paradigm permits the mitochondria to work even without a pH gradient, at low proton concentrations and without high trans-membrane potentials. This projection can be ratified in simple experimental systems. [Jagendorf's experiment (Jagendorf and Uribe, 1966) can be re-done by equilibrating mitochondria at pH 6 and then providing an external buffer of pH 4. The rates obtained above could be compared with an experiment where the mitochondria are equilibrated at pH 8 and then exposed to a buffer at pH 6. Such proton-gradients would be inadequate to power ATP synthesis (Manoj, 2017) per the chemiosmosis hypothesis even from

the initialized state. But yet, we may observe a net equilibrium-driven ATP synthesis, indicating the inapplicability of chemiosmosis hypothesis.]

* It can be explored if Complex III can set a one-electron scheme from CoQH₂ and H₂O₂ in the presence of oxygen+NADH. Further, this Complex's ability to bind ADP (as predicted by murburn scheme) can be explored.

* Cyanide is the most potent cellular respiratory toxin (Nelson, 2006) and therefore, it is perhaps the most important tool to investigate the mechanism of mOxPhos. The following experiments are suggested to further ratify the proposals/deductions of the current work on the impact of cyanide on cellular respiration and toxicity.

* In a simple reductionist system, the inhibitory role of cyanide in ATP synthesis can be traced. To a control reaction of ADP+Pi+superoxide, a test reaction of ADP+Pi+superoxide+cyanide can be compared.

* In respiring cells or mitochondrial suspension, (sub)micromolar levels of cyanide should be presented and consumption of oxygen and formation of DROS noted. While the binding-based perspective projects cell death and cessation of respiration, the new proposal predicts oxygen consumption leading to DROS formation (albeit at low levels).

* The catalytic role of cyanide radical/DROS can be traced by the incorporation of radio-labeled carbon in cyanide or radio-labeled oxygen. After exposing an experimental system to the molecular probes, the radio-labeled cyanate formation can be traced to confirm the radical pathway of CN/oxygen interaction. On the other hand, if CN binding to heme is efficient and irreversible, cyanate should not be formed.

* Similarly, if the efficient competitive ligand CO bound that effectively, one should not have DROS production and thereafter, a dissipation reaction of the equation scheme- $2\text{CO} + \text{OH}^* + \text{O}_2^{*-} \rightarrow \text{HCO}_3^- + \text{CO}_2$. Confirmation of the radical chemistry can be done with H₂S and CO, by tracing the radio-labeling of C/S and O atom within SO₂/sulfate and

CO₂/carbonate, respectively. It is expected that while H₂S would be highly metabolized, CO would be much lesser converted (as it is a better binder and poorer substrate). This could also be because the Hb in the blood would take up significant amounts of CO, and they do not have mitochondria to metabolize via DROS. (This would also explain the evolutionary reason for the higher affinities of Hb for CO, in comparison to Cox.) But per the murburn perspective, a mitochondrial suspension would surely oxidize CO to CO₂ and carbonate. Else, we cannot explain the reversal of toxicity in CO-exposed animals and individuals.

* Per the erstwhile purview, since cyanide has no bearing with Complexes I, II, & III (and since the purported ETC works without push or pull), these proteins should assist Complex V produce ATP via the “proton-based machinery”, if the old mechanisms were operative. So, at K_d (mM) concentrations of cyanide taken experimentally (Antonini *et al.*, 1971), only 50% of Complex IV is ‘bound by cyanide’. That would still leave 1:1-2:2-3:2-3:4-6:5-9 ratio of the complexes (I:II:III:IV:V:Cyt.c) in the mitochondrial suspension, and only a minimal or stoichiometric loss of activity should be noted. The new scheme predicts an overwhelming loss (practical cessation) in the ATP production ability of the system.

* A ‘cyanide-blocked’ system could be subjected to a favorable external pH gradient (say, 6.5 out to 9.5 in), quite similar to the demonstration by Mitchell’s group or a rhodopsin assisted ATP-synthesis via Racker-Stoeckinius type setups (Racker and Stoeckenius, 1974). While the erstwhile hypothesis does not seek inhibitions, the new perspective advocates cessation of fresh ATP synthesis.

* In any experimental system above, cyanide could demonstrate a maverick concentration-based modulatory effect. Say, at concentrations near nM levels of cyanide, murburn concept may result in an interesting observation that ATP synthesis could even be enhanced by a small fraction (say, up to 20 %).

* The multi-molecular Q-Cycle scheme of binding of a molecule each of CoQ, CoQH₂ and Cyt. c to the TM region of Complex III can be reinvestigated through experimental modeling and simulation. Molecular simulations of CoQ(H₂) diffusion in the phospholipid bilayer and binding to various proposed regions of the respiratory complexes should be revisited, owing to clear doubts that had been expressed in the field by renowned workers (Gupte *et al.*, 1984; Chazotte and Hackenbrock, 1989). Similarly, the diffusion and binding of cytochrome c and binding with Complexes III & IV should be simulated.

Summation

Since DROS catalyzes the synthesis of ATP in both reductionist and *in situ* systems, the case for murburn model is also strong because of the following-

1. Mitochondria (and each one of the four protein complexes) generate ROS, in the presence of NADH and oxygen (Ksenzenko *et al.*, 1992; Grivennikova and Vinogradov, 2006; Quinlan *et al.*, 2012; Bleier and Dröse, 2013).
2. The concentration of superoxide/peroxide (reactive oxygen species) produced is directly proportional to the NADH and oxygen presented (Murphy, 2009; Manoj *et al.*, 2019).
3. The magnitude of TMP and the amount of ATP synthesized is directly proportional to the ROS detected (Nicholls, 2004). ATP cannot be synthesized without the production of DROS.
4. The following equation, one which involves the intermediacy and formation of DROS better explains mOxPhos chemistry. It is an example that agrees with bond energetics, accounts for the reported higher efficiency of mOxPhos, connectivity of electron transport and ATP synthesis, overall phenomenology, and the role of Complex V (coupling agent serving protons in the steady state):

5. KMM's earlier works established that the non-active site and diffusible species mediated enzyme catalysis is an effective physiological modality. The prevailing perception that DROS are predominantly dangerous to life is not based on sound chemical or quantitative rationale (Ristow and Schmeisser, 2011; Mittler, 2017). In combustion, oxygen-centered radicals react among themselves to generate heat and form water. This is a common place phenomenon such as combustion or burning of wood. Therefore, an analogous but milder phenomena happens in cellular respiration. Heat generation in cellular respiration results from DROS reaction amongst

themselves, thereby confirming the observed outcomes and murburn model that NADH equivalents go into ATP formation or/and heat generation (Manoj, 2017; Manoj *et al.*, 2019).

Therefore, with respect to theoretical foundations (adherence to known chemico-physical laws) and current experimental data, the comprehensive aspects of the two models are summated below in Table 3.

Table 3: Holistic perspectives of the two models

No.	Aspect	RCPE model	Murburn model
1.	<i>Thermodynamics & kinetics</i>	Unviable because an inherently endergonic process (matrix-ward movement of pumped out IMS proton) cannot support another endergonic reaction (synthesis of ATP). Multi-molecular two-electron reaction scheme with CoQ cycle is too slow to explain for the overall reactivity.	The overall reaction is driven by water (O-H bond) formation in matrix and its subsequent efflux via aquaporins. The fast bi-molecular/radical reactions support the outcomes.
2.	<i>Working logic and architecture of the protein complexes</i>	On one hand, the deterministic scheme (with sequential or concerted array and ordered events) makes the system irreducibly complex and poses evolutionary constraints. On the other hand, the random distribution of components and non-sophisticated structures make the mechanism non-viable.	Simple stochastic scheme (unordered events with independent components) is favored by probability. Evolvable from a minimal set; distribution of components favorable.
3.	<i>Agreement with experimental information & overall coherence</i>	While several observations are inexplicable, some observations counter the model. Unviable with known chemico-physical fundamentals and inherently self-contradictory.	Most observations reported till date are adequately explained with no logical contradictions

Conclusions

Approximately thirty distinct predictions were seen to ratify the murburn scheme for mOxPhos, as opposed to the chemiosmosis explanation. While the RCPE model (or any proton motive force

or/and trans-membrane potential based explanation) appears highly misplaced, the murburn model does not pose major issues with known theoretical ideas and experimental facts. Of course, murburn scheme could also be proven wrong in the future, but for the same, the onus to propose a new explanation lies on the next one who can challenge it and prove that the murburn model is non-viable. Currently, the murburn model for mOxPhos needs more experiments and theoretical explorations to provide greater details of the working principles. For example- it is not clear as to what exact species are generated within the milieu, how does ADP/ATP binding influence the performance of Comp. V?, etc. It is envisaged that we would avail better insights in biology and medicine if all concerned warm up to the tenets of the new proposals.

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