Assessing the operational feasibility of ‘Z-scheme electron transport chain’ in chloroplasts

Kelath Murali Manoj¹*, Vivian David Jacob¹, Daniel Andrew Gideon¹, Abhinav Parashar¹,
Afsal Manekkathodi¹

*Corresponding author, ¹Satyamjayatu: The Science & Ethics Foundation
Kulappully, Shoranur-2 (PO), Palakkad District, Kerala-679122, India.
Email: satyamjayatu@yahoo.com

Abstract: We summarize salient components of the Z-Scheme electron transport chain within chloroplasts. Banking on the skepticism kindled by Robert Emerson’s reproducible observations, we explore the working of its various elements using updated information on the distribution of components within the chloroplast architecture, structure-function correlations of relevant redox proteins, thermodynamics, kinetics, and evolutionary principles. Through a set of simple models/simulations and a series of intriguing queries, we moot several theoretical premises and highlight evidences/arguments that question the physiological feasibility of Z-scheme. Finally, we also point to new avenues for furthering research in this field.

Keywords: thylakoids; electron transport chain; Z-scheme; chloroplasts; photosynthesis; photophosphorylation; photolysis; murburn concept

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An introduction to the Z-scheme of electron transport/transfer chain

The light reaction of photosynthesis generates the gaseous oxygen used by aerobes for cellular respiration and forms the primary source of ATP and NADPH that plants use for fixing CO₂, thereby sustaining the vast majority of life forms on planet earth. Assemblies of living systems (such as photosynthesizing chloroplasts) are at times complex to assemble and study in situ/in vivo. Therefore, reductionists extrapolate from experimental models in vitro/in silico or use the system in isolation and study them. Theoretical biologists aim to assemble/unify facts and simplify their explanation through abstract logical (both qualitative and quantitative) concepts, thus affording deeper understanding and greater predictability[1]. To explain bioenergetic routines, theoretical (and experimental) biologists have treated the pertinent redox metabolic machinery in mitochondria and chloroplasts under the traditional purview of ‘electron transport/transfer chains (ETC) [2-5]. Particularly, the theory of photosynthetic reactions occurring within chloroplasts is founded in affinity-based bindings (leading to relatively long-lasting ‘donor-acceptor complexes’) and Marcus’ outer sphere model electron transfers (ETs); and is detailed in modern textbooks under the caption of ‘Z-scheme’ ETC [6-8].

Emerson’s findings and the premise for skeptic’s challenge to the extant explanations for harvesting of sunlight by plants: The ‘Emerson enhancement effect’ (Figure 1, left panel) experiment [9] is a key turning point in photosynthesis research and a forerunner of the concept that two photosystems work in tandem within plant chloroplasts. Mitchell-Boyer’s “chemiosmotic rotary ATP synthesis” (CRAS) and Hill-Bendall’s multistep “Z-scheme of electron transport” (or ZS; including the photosystems’ discharge-charge cycles, Q Cycle for quinols at Cytochrome *b₆f* and the Mn-Complex mediated Kok-Joliot cycle or KJC for oxygenesis) are the currently standing explanations for the light reaction of oxygenic photosynthesis (Figure 1, right panel; Figures 2 & 3), also known as Photolysis-Photophosphorylation (Pl-Pp) [8]. Robert Emerson’s findings is reproducible in diverse photosynthetic systems [10]. We find that the ZS-KJC paradigm is incompatible with key aspects of Emerson’s findings. As per the Z-scheme, Photosystem I (PS I or P700, excited at far-red light) cannot photolyze water because it lacks the water-splitting or oxygen-evolving complex (WSC/OEC) or MnComplex. Then, it is inexplicable why Emerson observed that oxygen

evolved in the instance when the system was exposed to only far-red light. Therefore, the extant KJC model's doctrine that oxygen evolution occurs only at WSC of Photosystem II (PS II or P680, excited at red light) seems misplaced. From another angle- without PS II, PSI would be dysfunctional because it cannot directly drain electrons from water. Therefore, the ZS-KJC explanation does not afford independent functioning and/or evolution of PS I. Further, Z-scheme proposes an elaborate and serially connected route for the travel of electrons derived by photolysis of water, via the photosystems and redox proteins, via multi-molecular complexes, to the ultimate destination of NADP^+ . This is destined to be a very slow and practically 'low probability' process, by any standard of imagination.

Figure 1: Emerson's experiment and the origin of Z-scheme. The left panel shows salient highlights of the Emerson experiment, as taught the world over today. On the right: the minimal components and course of the electron flow in Z-scheme, as proposed and promulgated since the 1960s. The native P680, excited P680*, native P700, and excited P700* form the four defining points of the Z-scheme, in the electron transfers from water, via a vitally deterministic trans-membrane journey, to reduce NADP^+ .

Figure 2: A ‘visualization of the actual path’ and sequential/serial transfers in the Z-scheme. The stoichiometry and the statistical location of components are shown whereas the details of proton pumps are omitted. Dotted arrows signify movement in space whereas continuous arrows are ETs within a protein complex or within donor-acceptor proteins’ complex. (Fd = ferredoxin, FNR = Fd-NADP reductase) Trans-membrane proton potential (TMP) across the thylakoid membrane is depicted on the left with –ve and +ve signs.

Figure 3: A depiction of the expanded temporal and energetic aspects of Z-scheme. The relative redox potentials (on the Y axis, which was earlier shown along the X axis, as in Figure 1) of listed components are merely a rough depiction, and the boxed entries (water and NADPH) and K-J/Q cycle components are not included within the purview of the Y axis. The number of breaks within a phase arrow indicates the approximate number of currently presumed transient relay centers or intermediates. The cyclic intermediates of the photosystems’ reaction centers (RC) have been expanded to show the details with respect to photo-excitation, emission and electron recharge. Four excitation each (at PS II and PS I) are required to power the electron flow from water to NADPH.

Essential details of the Z-scheme ETC: The minimal rudiment of Z-scheme ETC was originally formulated in 1960 by Robin Hill & Fay Bendall and its tradition was continued by researchers in the field thereafter [11]. As shown in Figure 1, the term Z-scheme stems from the temporal change of redox potential of an electron, when it surges from the native/stable P680 to the excited P680* (the top rightward horizontal stroke of Z), then declines via several intermediates to the native/stable P700 (the diagonal leftward stroke of Z), only to surge again to the excited P700* (the bottom rightward horizontal stroke of Z).

A more elaborate depiction of the spatio-temporal and energetics layout of the Z-scheme is shown in Figures 2 and 3. The electron(s) must first travel from the water molecules bound to the WSC (water splitting complex or MnComplex, located in the thylakoid lumen side) to the membrane-embedded RC₂ (reaction center) of Photosystem II (PS II) protein complex. From there, electrons are relayed via a series of fixed and mobile agents [of which plastoquinone (PQ) is the most salient] to reach the trans-membrane region of Cyt. *b₆f*. Then, electrons are ‘bifurcated’, one part of which is relayed through redox centers to heme *f* (present in the lumen), and collected by plastocyanin (PC). PC relays the electron to the luminal side of Photosystem I (PS I). Thereafter, the electrons traverse through the PS I into the stromal side, to be picked up by ferredoxin (Fd) and finally, used to reduce NADP⁺ by the Fd-NADP reductase (FNR) enzyme. It was earlier believed that while the electron transport occurs, PS II and Cyt *b₆f* complexes also act as proton pumps [6], but the later consensus appears to be that only the latter protein complex serves in this capacity. Such pumped out protons ultimately constitute a proton motive force or serve as triggers to phosphorylate ADP via the rotary ATP(synth)ase. Thus, Z-scheme is a concept which mandates that electrons that arise from water (bound at the oxygen evolving MnComplex of PS II) must pass through a series of redox centers located in the thylakoid lumen, membrane and stroma and also undergo periodical excitations at P680 and P700, before they reach their destination at FNR, to finally reduce NADP⁺. In toto, PS II-Cyt. *b₆f* ensemble breaks water (liberating oxygen and electrons) and pump the stromal protons into thylakoid lumen, while PS I-FNR ensemble pack the electrons into NADPH, and ATP(synth)ase complex uses the luminal protons to make ATP in stromal side. From the energetic perspective, there are three downhill ET phases (as duly labeled in Figure 3) and an electron from water needs to be powered by two photons to travel all the way to NADP⁺. Therefore, a total of 4 + 4

photon excitations (or zaps) each are required for the formation of a molecule of oxygen or two molecules of NADPH. In the Z-scheme, water molecules within the thylakoid lumen serve as the source of electrons and the NADP^+ molecules present within stroma are the electron sink. Within the Z-scheme, the Kok-Joliot oxygenesis cycle is a salient component of the serial electron transfer mechanism, which supposedly occurs at the MnComplex of PS II and a brief excerpt is shown within the left inset of Figure 3. In this view, MnComplex must proceed through a sequential and cyclic change of oxidation states from S(0) to S(+1), S(+2), S(+3) and S(+4). The most important aspect is that while electrons (and protons) can be released at various (or stipulated) time points from the MnComplex to the reaction center (RC) of PS II, O_2 is released only at a unique instance [S(+4)] in the mechanistic cycle. This implies that diffusible reactive oxygen species (DROS) formed in the chloroplast should be deemed as toxic wastes of metabolism. Another important aspect of the Z-scheme is the recycling of reduced quinones, which purportedly occurs at Cyt. *b₆f* (central inset of Figure 3). Q-cycle involves the deterministic formation of tetra-molecular complexes lasting millisecond timeframes. In other words, the four electrons from two molecules of reduced plastoquinone (PQH_2) are used to reduce a molecule of PQ and two molecules of PC, which subsequently dissociate from Cyt. *b₆f*. Supposedly, during these processes, the latter protein (and/or PQ) also pumps out protons into the stromal side [7]. Thus, chemiosmotic rotary ATP synthesis (CRAS) and Z-scheme (ZS) of electron transfer are the acclaimed/textbook view for oxygenic photosynthesis [6, 7, 8].

Current status appraisal: The explanations for oxygenic photosynthesis were based on ideas unleashed in mid-20th century, many of which were questioned and disproved. Further, the architectural details of chloroplast and structure/distribution of proteins therein were lesser known than the current times. Furthermore, redox enzyme mechanisms were confined only to active-site mediated electron transfers/processes. Our studies in the last two decades have focused on the theoretical and experimental aspects of diverse porphyrin, Fe-S and flavin based biological redox catalysts. To explain some anomalous observations, we have expanded the purview of classical enzymology and proposed a non-specific diffusible species mediated ET scheme called murburn concept [12]. This mechanistic principle was applied for explaining the holistic outcomes in select extracellular and peroxisomal soluble redox enzymes first; and later on, adapted to explore membrane-based electron transfer chemistry of microsomal xenobiotic

metabolism and mitochondrial oxidative phosphorylations [13-18]. Further, we had also questioned the viability of intricate electron transfer chains/circuitry in/around mitochondrial membranes, quoting a wide variety of arguments and observations [19] and provided a thermodynamically and kinetically facile alternative reaction paradigm [20]. Considering these realities and latest evidences, we find ample reasons to critically examine the CRAS-Z-scheme model's viability for explaining the light reaction of chloroplastid photosynthesis.

Assessing/dissecting the Z-scheme ETC with some critical queries

We shall pose some fundamental queries that could enable a critical inspection of the Z-scheme, with respect to the feasibility of proposed reactions in real space/time and adherence to known fundamentals of thermodynamics, kinetics, and other bio/chemical logic.

1. Is the CRAS-ZS mechanism viable with respect to the fundamental principles of electrochemistry? Figure 2 can be redrawn via a simpler two-dimensional perspective, to draw out the movement of electrons with respect to the membrane, as shown in panel B of Figure 4. As per the Z-scheme, photo-energy driven proton-pumping establishes a net negative charge at stromal side and a net positive charge at lumen side, forming the 'potential generating, working machine logic' of chloroplast. As per this mechanism, four electrons from water in the lumen undertake a one- or two- electron journey via PS II, Cyt. *b₆f* and PS I to finally pass into the stromal side via Fd. The overall setup can be compared with electrolysis, which employs electrical poles (cathode and anode) to enable charge separations and redox reactions (Panel A of Figure 4). In an electrolytic setup, negatively charged particles move towards the positively charged electrode (or anode). Analogously, lumen is the positively charged region and therefore, elementary electrochemistry would dictate that electrons move from stroma to lumen in the steady state. Further, there are little rationales for the bi-directional movement of protons (positive charge) across the same membrane, in closed-pot scheme that Mitchellian paradigm solicits. In conjunction with the facts that- (i) there are no long-distance pull or push mechanism for electron movements, (ii) micrometer-dimensioned organelles cannot sustain proton pumps [as the physical properties of water would not allow adequate protons within the ensconced milieu[16, 17, 21, 22] and (iii) stark pH gradients are not observed in physiological ambience-

the critical dissection above suggests that the CRAS-ZS view of chloroplast physiology is questionable.

Figure 4: Comparison of usual electrolytic cells to Z-scheme mechanism. A half-arrow shows the transfer of an electron whereas a full arrow is the transfer of electron pairs. As seen within the Z-Scheme (panel B), there are both one and two electron transfers. After starting off from the lumen, the electrons take refuge in the membrane, to return to the lumen again (carried on PC), ultimately traversing the membrane into the stromal side. The lumen is maintained at a positive charge because protons are pumped into it. At the same time, protons are also supposed to move back into the stroma spontaneously. These concepts counter the fundamental awareness based on normal electrochemical systems (panel A), as such a Z-scheme system cannot have sustained charge-balance dynamics within the closed purview of Mitchellian model. The thunderbolt sign within the membrane designates the powering element.

2. Does Z-scheme fit into the criteria for simple electron transfer cascades? When correlated in time with the energetic stature of the electron, the conventional Z-scheme picture can be elaborated to Figure 3. The three downhill reactions shall be addressed first. Phase I ET is currently supposed to involve the intermediacy of at least two relay centers; Water $\rightarrow$ MnComplex $\rightarrow$ Tyr161 $\rightarrow$ P680⁺. Phase II involves several relay centers, of which the salient ones are: P680* $\rightarrow$ Pheophytin $\rightarrow$ bound & free Plastoquinone molecules $\rightarrow$ heme and FeS centers of Cyt *b₆f* complex $\rightarrow$ Plastocyanin $\rightarrow$ P700⁺. Finally, Phase III is supposed to involve several relay centers and the key elements are: P700* $\rightarrow$ ChlA₀ $\rightarrow$ Phylloquinone $\rightarrow$ Three Fe-S clusters $\rightarrow$ Fd $\rightarrow$ FNR $\rightarrow$ NADP⁺. It must be noted that all phases start with a stable reduced species that get oxidized, leading to a series of electron transfers or relays, or redox cycles. Then, there are also two photon-induced obligatorily uphill excitation steps (Zap I = P680 to P680* and Zap II = P700 to P700*).

Let us trace the ETC route by setting an electron relay into motion by the excitation of P680 with a photon of suitable wavelength. (That is- we operate Zap 1 alone.) At the end of a suitable time

point, let us assume that an electron would have traveled through Phase I and reached the end of Phase II (without yet going all the way into the final acceptor of $P700^+$). Repeated operation of Zap 1 would not enable any further non-cyclic electron transfers because Phase II cannot be completed and electron(s) cannot get transferred to $P700^+$ (because Zap 2 has not occurred) and onwards to $NADP^+$ thereafter. On the other hand, if we had operated Zap 2 alone, one electron would have only completed Phase III. Repeated operation of Zap 2 would not lead to any further relay even in this phase because the oxidized $P700^+$ cannot get excited or give electrons any further. So, we see that for the prevailing Z scheme to be operative (for the complete one-electron travel all the way from water to $NADP^+$, completing all three phases), two zaps would be required to synchronize (one photon each would be required for zapping each one of the photosystems), for overcoming the breaks in continuity within the ‘linear relay’. Restating, these two “relay-breaking” discharge-charge sequences at the photosystems (namely: $P680^* \rightarrow P680^+ + e \rightarrow P680$ and $P700^* \rightarrow P700^+ + e \rightarrow P700$; Figure 3) arise because-

1. Electron relay from water cannot charge an electron-satisfied P680 and/or an electron deficient $P680^+$ cannot get photo-excited to $P680^*$. (Let’s call this as Gap 1.)
2. $P680^*$ cannot transfer electrons to an electron-satisfied P700 and/or an electron-deficient $P700^+$ cannot get photo-excited to $P700^*$. (Let’s call this as Gap 2.)

This means that we have to write both the oxidized and reduced forms of the two key photo-excitabile components (P680 & P700) of the ETC in the diagram (Figure 3) as a pre-requisite for the three downhill phases and two photon-zaps to ‘connect’ and work repeatedly. Therefore, the elements cannot be given the same representation as a routine electron transfer chain (Figure 5), as this is a disrupted sequential non-cyclic process. To illustrate the point further, a comparison can be given here. The purported mitochondrial oxidative phosphorylation (mOxPhos) relay of “ $NADH \rightarrow \text{Complex I} \rightarrow \text{CoQ} \rightarrow \text{Complex III} \rightarrow \text{Cyt. } c \rightarrow \text{Complex IV} \rightarrow \text{Oxygen}$ ” could be taken as a theoretical example of ETC (if such a concept worked physiologically). In this example, the primary source of electrons (NADH) is taken in the reduced state and all the subsequent elements of the ETC (including the final acceptor of electrons, oxygen) are taken to be in the oxidized state. This is the requisite for a continuous downhill electron transfer process and the given elements of the ETC can “theoretically” sustain the ‘stringed’ electron relay by alternating between two redox states, upon receiving and giving electrons. One can see that the

requirement for depicting such feasibility is that all elements (except the primary electron donor) within the relay must be oxidized at the commencement of the ETC. If we do the same exercise for the PI-Pp ETC, we need to write ChlP680⁺ as the terminal electron acceptor for Phase I. But if we do so, it will not allow us to incorporate the photo-excitability element of PI-Pp into the scheme any further. Therefore, the more factual Z-scheme (as shown in Figure 3) enables us to visualize the chinks of the overall redox process. Further, we can see that the Z-scheme incorporates the cyclic processes such as K-J cycle (at MnComplex), P680 cycle (at PS II), Q-cycle (at Cyt. *b₆f*), P700 cycle (at PS I) and linear relays such as Q_b to PQ (at PS II), Phylloquinone to FeS_x (at PS I), Fd to FNR (in stroma). Furthermore, low redox gradients and distances bigger than 10-12 Å are deemed to be of low probability to constitute a feasible component of the overall flow (from 1-2 to 18-19). It can also be seen that several steps of Z-scheme do not agree with the requirements of a functional redox relay chain and would be fraught with connectivity issues, as listed in the second part of Figure 5. For example: (i) the multitudes of chlorophyll and carotenoid molecules randomly scattered throughout could shunt the relays & (ii) synchronizing one or two electron relays to one, two or four electron cycles is a practically impossible task.

Figure 5: A schematic representation of a functionally propagating ETC compared to the chain-breaking aspects of Z-scheme. In a viable ETC (given in the left panel) starting at time T_0 , movement of electron from a source (say, via three intermediates) to a sink is represented with the primary donor written in a reduced state and the rest of the components present in oxidized states. Further, they must be present at optimal distances and pose suitable potential gradients. The remaining steps (from time T_1 to T_4) deal with the sequential/serial propagation of the electron transfer chain, with the bottom step as the final state. In the temporal relay process, the intermediates periodically shift their oxidation states. In the Z-scheme ETC (right panel), there are hindrances to a smooth electron relay. In the first two rows, variable redox/energy states of intermediates within the chain retard the electron relay. Further, the intermediates are at times presented without favorable gradients (3rd row), there could be shunting intermediates present in between (4th row), separation is seen by non-optimal distances (5th row) and the

relaying agent could be absent altogether (6th line). For examples of contextual relevance within the Z-scheme ETC, please refer the data given within Table 3 and discussions within the text.

3. Can Z-scheme's serial/sequential connectivity actually explain Emerson's enhancement experiment? Emerson first reported the reproducible observation that the combined photo-excitation of PS I and PS II gives significantly higher yields (as measured by oxygen evolution) than the added outcome of independently (separately) activating PS I and PS II with the corresponding monochromatic light[9]. While the Z-scheme explanation not only fails to explain why oxygen evolves even with only PS I excitation (as only PS II's Mn-Complex is supposed to liberate oxygen), it also doesn't account for the enhanced yields obtained with combined excitation of PS I and PS II. Physiological evolution rate or yield of oxygen (the criterion Emerson used to determine photosynthetic rate) can be taken as equivalent to the electron transfer rate from water to NADP^+ , i.e. the functionalism of ETC. Electron transfers via multiple redox centers can be compared to the flow of fluids through a plumbing network (a simple analogy) or conduction of charges (current) in conventional electrical circuitry (a sophisticated analogy). We know that there are at least seven major components or pit-stops of the Z-scheme ETC (PS II, PQ, Cyt. b_6f , PC, PS I, Fd, & FNR). In the first analogy, each component can be considered to a pipeline of defined width and length. In this case, the oxygen yield ($\approx$ electron flow) is compared to fluid flow through pipelines. Here, the combined output of any number of elements connected in series is limited by the component with the slowest flow rate within the system. In the second analogy, each component can accept and donate electrons and thus, each component needs to conduct or relay electrons. Therefore, they can be envisaged to have the electrical properties of capacitance and conductance, respectively. For simplicity, we shall assume that there are only two components (say, Photosystems I & II) that contribute to fluid flow rate or electrical resistance or electrical capacitance. Table 1 can allow the reader to visualize and simulate the result of flow rate or overall capacitance/conductance obtained in the independent or serial or parallel functioning, with any randomly assumed values. It can be seen that in both analogies, only a parallel functioning of components approaches the physiological outcomes. Therefore, it is imperative that a parallel configuration (stochastic interaction of components, as afforded by murburn scheme) is physiologically operational, and this key finding is opposed to the serial linking format espoused in Z-scheme. Therefore, Z-scheme cannot stake

claim to quantitatively or qualitatively account for the Emerson enhancement experiment, i.e. the physiological synergy of PS I+PS II.

Table 1: Analogy for serial and parallel connectivity for the major two redox-active components (PS I and PS II systems) of the chloroplastid photosynthetic machinery.

Analogy	Powering element	Criteria	Serial connectivity	Parallel connectivity	Expected Result	Remark
Fluid flow rate in pipelines	<i>Pump</i>	<i>Flow rate</i>	(Rate is limited by one component)	(Rate is equal to the individual terms added.)	(Flow rate should at least equal the individual terms added.)	Parallel functioning gives higher flow rate than serial linking.
Current flow in electrical circuit	<i>Battery</i>	<i>Conductance</i> (inverse of resistance, R)	$(1/\Sigma R_i)$	$(R_1 \times R_2 / \Sigma R_i)$	(Current flow should be more than the individual terms added.)	Parallel networking explains the higher flow of electrons, for varied capacitances.
		<i>Capacitance</i>	$(\Sigma 1/C_i)$	(ΣC_i)	(Effect of capacitance varies with respect to the components.)	

4. Is Z-scheme compatible with the architecture of chloroplasts and does it present spatio-temporally operable/probable machine logic? Now, the sequence of ET events boils down to two series of concomitant and distinct but interdependent (and repeated) temporal electron transfer operations centered at the two loci (PS II and PS I), as captured in Figure 6 below. The spatio-temporal essence of the same is captured by two operations:

Operation 1 (Overall uphill steps), Zap & Discharge: (i) P680* to PC (Zap I & Phase II) AND (iii) P700* to NADP⁺ (Zap II & Phase III) **AND**

Operation 2 (Overall downhill steps), Charge: (iia) PC⁻ to P700⁺ (Charge II) AND (iib) Water to P680⁺ (Phase I and Charge I)

Figure 6: A breakdown of the electron transfer phenomenology within the purported “Z scheme” of Pl-Pp ETC, with a spatio-temporal perspective. The transfer of electrons from stable (reduced) participants to yield stable (reduced) products within a reaction leg is shown. Since the reaction turnover is in millisecond timescale, stability is a time period that is significantly comparable to this scale.

For the evolution of a molecule of oxygen, the setup must go through the precise sequential and iterated orchestrations of 4 cycles: Operation p1 + Operation p2 + Operation q1 + Operation q2 + Operation r1 + Operation r2 + Operation s1 + Operation s2 steps; where p, q, r and s are sequential rounds of one-electron equivalents (totaling four) entering the ETC, through the MnComplex. (This, in turn, is triggered by electron losses at reaction center of P680 and P700 by precisely coordinated zaps.) Very clearly, the most crucial bottleneck step in the overall acyclic sequence is the dynamics of Phase II, leading to the production and transport of $PC^{\bullet}$, which ultimately governs the connectivity of operations between the spatially disconnected reaction centers of PS II and PS I. [This step is akin to cytochrome *c* serving electrons from Complex III to Complex IV, in the mOxPhos CRAS-mechanism. We have already demonstrated that such

mechanistic processes are untenable [21]. For synchronizing ETC and oxygenesis cycles (as explained with respect to Figures 2-3), let us ideate a simple and favorable scenario. Let it be given that a chloroplast has 8 molecules of water bound to 4 WSC units and let there be 4 molecules each of PS II and PS I. It is also assumed that these are present along with an unlimited or optimal amount of the necessary redox-relay intermediaries and final acceptor molecule, NADP^+ . Let us also suppose that they are arranged in an optimally well-knit circuit. Let us now assume that both push-pull mechanisms of electron-transfer are operative. These ideas are represented within Table 2 and the details therein are discussed below.

Table 2. Idealized temporal phasing of intermediates and electron flow through various components of the Z-scheme vis a vis Kok-Joliot oxygen evolution cycle: A minimalist and stoichiometrically idealized model for the first four flashes (leading to a 16-electron reaction) in a chloroplast with four modules each of PS I and PS II is shown. This scheme has been visualized starting from Kok's proposed ratio of $S1:S0 = 3:1$ (wherein at the initial state, $S0 + S1 = 100\%$; $S2 = S3 = 0\%$). Discounting misses and double-hits, the 3rd and 4th Zaps' oxygen evolution corresponds to initial $S1$ and $S0$ populations, respectively.

Cycle No.	State	WSC Status	PS II Status	PS I Status	Products
	T0 Rest	$\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{H}_2\text{O}$	P680, P680, P680, P680	P700, P700, P700, P700	Nil
Operation p1	T1 1st Zap	$\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{H}_2\text{O}$	P680*, P680*, P680*, P680*	P700*, P700*, P700*, P700*	Nil
	T1 1st Discharge	$\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{H}_2\text{O}$	P680 ⁺ , P680 ⁺ , P680 ⁺ , P680 ⁺	P700 ⁺ , P700 ⁺ , P700 ⁺ , P700 ⁺	2NADPH
Operation p2	T1 1st Charge	$\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{OH}^*$	P680, P680, P680, P680	P700, P700, P700, P700	(2H ⁺)
Operation q1	T2 2nd Zap	$\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{OH}^*$	P680*, P680*, P680*, P680*	P700*, P700*, P700*, P700*	Nil
	T2 2nd Discharge	$\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{O}$, $\text{H}_2\text{O}-\text{OH}^*$	P680 ⁺ , P680 ⁺ , P680 ⁺ , P680 ⁺	P700 ⁺ , P700 ⁺ , P700 ⁺ , P700 ⁺	2NADPH
Operation q2	T2 2nd Charge	*HO-O, *HO-O, *HO-O, $\text{H}_2\text{O}-\text{O}$	P680, P680, P680, P680	P700, P700, P700, P700	(2H ⁺)
Operation r1	T3 3rd Zap	*HO-O, *HO-O, *HO-O, $\text{H}_2\text{O}-\text{O}$	P680*, P680*, P680*, P680*	P700*, P700*, P700*, P700*	Nil
	T3 3rd Discharge	*HO-O, *HO-O, *HO-O, $\text{H}_2\text{O}-\text{O}$	P680 ⁺ , P680 ⁺ , P680 ⁺ , P680 ⁺	P700 ⁺ , P700 ⁺ , P700 ⁺ , P700 ⁺	2NADPH
Operation r2	T3 3rd Charge	$\text{H}_2\text{O}-\text{H}_2\text{O}$, $\text{H}_2\text{O}-\text{H}_2\text{O}$, $\text{H}_2\text{O}-\text{H}_2\text{O}$, *HO-O	P680, P680, P680, P680	P700, P700, P700, P700	3O ₂ (2H ⁺)
Operation s1	T4 4th Zap	$\text{H}_2\text{O}-\text{H}_2\text{O}$, $\text{H}_2\text{O}-\text{H}_2\text{O}$, $\text{H}_2\text{O}-\text{H}_2\text{O}$, *HO-O	P680*, P680*, P680*, P680*	P700*, P700*, P700*, P700*	Nil
	T4 4th Discharge	$\text{H}_2\text{O}-\text{H}_2\text{O}$, $\text{H}_2\text{O}-\text{H}_2\text{O}$, $\text{H}_2\text{O}-\text{H}_2\text{O}$, *HO-O	P680 ⁺ , P680 ⁺ , P680 ⁺ , P680 ⁺	P700 ⁺ , P700 ⁺ , P700 ⁺ , P700 ⁺	2NADPH
Operation	T4	$\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{OH}^*$, $\text{H}_2\text{O}-\text{H}_2\text{O}$	P680, P680, P680, P680	P700, P700, P700, P700	O ₂ (2H ⁺)

<i>s2</i>	<i>4th Charge</i>	H ₂ O-OH*, H ₂ O-H ₂ O	P680, P680	P700, P700	
4 cycles	4x2x4 =	[4WSC];	[4ChlP680];	[4ChlP700];	4O₂, 8H⁺
	32 Zaps	8H₂O	16 photons (λ1)	16 photons (λ2)	8NADPH

Disregarding the time-frame needed for several zaps, electron-relays and molecular transports, let us take an account of the overall process. There are ~20 redox active species in the Z scheme with approximately similar number of distinct intra- or inter- molecular electron transfer steps (from H₂O to NADP⁺). Each element can be in oxidized or reduced state and therefore, the chance of finding the particular relay agent in the suitable redox state is 1/2. So, the sequential scheme's overall crude score (within all available sample space) would be $(1/2)^{20}$ or less than one in 33 million. [[Introducing complexities into this analysis is vain exercise but we shall indulge in it, albeit briefly. Let's consider the four key components of the ETC, "WSC-PS II-PS I-2NADP⁺". With respect to the bound ligands and without considering symmetry and protonation aspects, a WSC can exist in a total of 5 states (H₂O-H₂O, H₂O-OH*, H₂O-O, *HO-O, O-O) of which four can favor the reaction. Each one of the two photosystems can be in three unequal states- native, excited and discharged, of which only one can favor the reaction. Similarly, the final leg can also be in three states: NADP⁺, NADP* and NADPH, and out of these, two can support the reaction. Now, the fruitful reaction of the sequentially wired "WSC-PS II-PS I-2NADP⁺" system would be: $(4/5) \times (1/3) \times (1/3) \times (2/3) = 8/135$ or $< (1/2)^4$. Now, if we factor in the remaining elements of ~ 16 redox species (with each one of these transients having an oxidized and a reduced state), the probability of favorable outcome within the overall sample space would be $(1/2)^{16} \times 8/135, \approx (1/2)^{20}$. If we consider that intricately connected redox centers within a particular protein complex are not subject to such probability calculations, that still makes the "H₂O-WSC-P680-Pheo-Q_{AB}-PQ-Cyt.*bf*-PC-P700-A₀₁-Phy-FeS_{XAB}-Fd-FNR-NADP⁺" serial connectivity a formidable 15-component relay. This would still have a highly fastidious probability score of $(1/2)^{15}$ or less than one in a million.]] It is seen that in the above scenario (with a 4 PS I + 4 PS II system setup, as simulated in Table 2), the synchronized reactions have very low probability. If we accept the Kok-reasoned scenario (at T0 time) that one out of four WSC centers is in the native state and three are in a partially excited state, then we need to have all the remaining centers go through the precisely coordinated scheme of events with each zap, discharge and recharge routine. To beat the probabilistic odds, this scheme would

require immaculate connectivity (or wiring) and ordered placement of the participants. However, neither the process of morphological differentiation of leaf tissue nor the final structures of various leaves show any such anatomical or structural evidence. It is improbable that such cycles must also be synchronized with the electron transport relays. This requisite becomes even more difficult to meet when it is noted that- (i) the spatial arrangement is not in a crystal lattice, (ii) many crucial components of the scheme are freely mobile, and (iii) the incident light beam could have diverse intensities, angle of incidence, wavelength spread, etc. The locations of most chlorophyll molecules are an outcome of a stochastic distribution. Further, the chlorophylls are not tethered to the lipid layer or the embedded protein via any covalent links either [23]. How could such leaves with a rather ‘random architecture’ function in reality, with a deterministic mandate for electron flow? This is when- (i) the electron needs to go both uphill and downhill (with respect to energy terms), (ii) there are connectivity gaps, (iii) there are both one and two electron exchanges, and that too, up and down the membrane and across macroscopic phases, disregarding the trans-membrane potential, (iv) the overall process is essentially uphill, (v) the ET reactions are not known to be specific, as several molecules and dyes are known to give/take electrons from diverse molecules of the purported ETC [24].

5. Does the Z-scheme do justice to the structure-function correlation and distribution of the chloroplast proteins and reaction components? A curious scholar may pose the following queries: Why should an electron take such a devious route from the WSC, up and down and across the membrane via several proteins, travel within the membrane, only to finally traverse the membrane to go into stroma, to reduce NADP^+ ? If reduction of NADP^+ and synthesis of ATP was the purpose, why could two separate proteins not achieve this purpose, aided by light? Why should one-electron relays be seen in chloroplasts along with two-electron pit-stops when ATP and NADPH syntheses are purportedly two-electron reactions? How can the electron transport chain function as a fecund relay when oxygen and DROS (and chlorophylls/carotenoids) are practically omnipresent? Why are unfavorable redox potential gradients in the way and why are DROS produced at the WSC side of PS II, Cyt. *f* side and $\text{FeS}_{\text{A-B}}$ side of PS I wherein least favorable redox potential gradients are noted? Why are the numerous randomly distributed (non-covalently bound) carotenoid and chlorophyll molecules noted in the crystal structures of the various thylakoid proteins? Why should two Photosystems be present and why are the activation

wavelengths at the end of the visual spectrum? Why are the LHC located so distant and how can the energy of photons be relayed so far, across membrane and protein structures, through an ‘amorphous and heterogeneous lattice’? How could quinones access redox centers move freely through the membrane and protein maze? Why should the protein complexes have large luminal and stromal extensions (particularly, PS II and NDH, which have no attributions whatsoever, given by the Z-scheme!) and why are the membrane embedded regions full of crypts and crevices, exposing the redox centers? The extant model does not give any pointers with respect to such queries.

6. Can the Z-scheme fit into a viable thermodynamic and kinetic purview? In our recent manuscript [25, 26], we have already presented novel thermodynamics/kinetics-based arguments that discredit the Z-scheme stoichiometry, which purportedly shows “miraculous efficiency”, exceeding 100%. Analysis of the energetics of the PS II and PS I reaction also showed that if the Z scheme were to function physiologically, it would require a 3 fold ratio of PS I to PS II. Quite simply, this is not the physiological case, as PS II usually exceeds PS I [27]. Further, we have also analyzed the roles of plastocyanin (PC) and ferredoxin (Fd) in chloroplasts [28] and found that the reaction kinetics, protein structure and mutation studies do not support the ‘specific electron carrier’ role for PC/Fd. The following discussion presents several key thermodynamics and kinetics arguments presented, other than the ones already cited. In this regard, changing the analysis in point 3 above by introducing weights for the stability time-frames of individual components (or by increasing the number of hits in time or even taking out a few components) does not significantly enhance the appeal of the Z scheme, because the espoused mechanism is obligatorily ordered and sequential. That is, the purported scheme is A to B to C to D.... all the way till Z; and not via a haphazard manner like A to C to D to B....Z or shunted fashion like A to C to Z). Nevertheless, let’s now analyze the time factor. The timescale of water-splitting at MnComplex is in the order of milliseconds and the synthetic rate of NADPH/ATP also occurs in milliseconds. This is when photocatalysis at reaction centers is in the order of nano- to pico-seconds. Further, as per the Z scheme, there are several reactions in the millisecond scales (Table 3).

Table 3: A compilation of the comprehensive spatio-temporal and energetic coordinates of the Z-scheme.

No.	Step	From (mV)	Distance [#] (Å)	Time [@]	To (mV)	Yield/ log k_{ET} (kJ/mol) (s ⁻¹)	Probability
H₂O-PS II-PQ (K-J Cycle + PS II Cycle)							
1	2H ₂ O to MnComplex	815 (4e)	2.2	~1 ms	820 (1e)	-0.5/12	
2	MnComplex to Tyr161	820 (1e)	4.6	≤1 ms	1000 (1e)	-17/11	
3	Tyr161 to RC ₂ Chl <i>a</i>	1000 (1e)	13.5	<30 μs	1190 (1e)	-18/6	
4	RC ₂ Chl <i>a</i> to Pheophytin (via P _A = ChlD ₂)	-620 (1e)	10.2+10.6	< 35 μs	-530 (1e)	-8.7/1	Low
5	Pheophytin to Q _a	-530 (1e)	13.8	<250 ps	-140 (1e)	-38/6	
6	Q _a to Q _b	-140 (1e)	17.8	≤0.6 ms	-60 (1e)	-7.7/3	Low
7	Q _b to PQ	-60 (1e)	17.0	<20 ms	80 (2e)	-27/4	Low
PQH₂-Cyt. <i>b₆f</i>-PC (Q-Cycle)							
8	PQH ₂ to FeS	80 (2e)	26.4	<20 ms	310 (1e)	-44/-1	Low
9	FeS to Heme <i>f</i>	310 (1e)	26.9	<1 ms	365 (1e)	-5.3/-3	Low
10	Heme <i>f</i> to PC	365 (1e)	16.7	<0.15 ms	375 (1e)	-1/3	Low
PC⁻-PS I-Fd (PS I Cycle)							
11	PC ⁻ to RC ₁ Chl <i>a</i>	375 (1e)	~25	~3 ms	420 (1e)	-4.3/-2	Low
12	RC ₁ Chl <i>a</i> to Chl A ₀ (via P _A = Chl A)	-1300 (1e)	11.8+8.5	<100 μs	-1100 (1e)	-19.3/2	Low
13	Chl A ₀ to Phylloquinone	-1100 (1e)	7.9	~20 ns	-850 (1e)	-24/9	
14	Phylloquinone to FeS _x	-850 (1e)	13.4	<100 ns	-700 (1e)	-14.5/6	
15	FeS _x to FeS _a	-700 (1e)	13.7	<100 ns	-550 (1e)	-14.5/5	
16	FeS _a to FeS _b	-550 (1e)	10.7	<0.5 μs	-600 (1e)	4.8/6	Low
17	FeS _b to Fd	-600 (1e)	~9.0	~1 ms	-410 (1e)	-18.4/9	Low
Fd⁻-FNR-NADP⁺ (FNR reaction)							
18	Fd ⁻ to FAD	-410 (1e)	9.1	~1 ms	-380 (2e)	-5.8/8	
19	FAD to NADP ⁺	-380 (2e)	3.5	~1 ms	-320 (2e)	-12/11	

[#]average of (edge-edge) and (center-center) distances; [@]maximum time frame of reaction.

Distances: MnComplex to water [29]; Q_b and Q_c near PQ binding site [30]; PS II [31] Cyt. *b₆f* [32], PS I [33]; Fd & FNR [34].

Redox potentials: [11, 35].

Reaction timeframes: [11].

Yield/log k_{ET} : Computed in this work; (for methodology, refer- [36])

Remarks: Low when yield is positive or log k_{ET} is less than 3. The Q-cycle reaction steps have low feasibility assessments for the quinone reduction steps also for plants and cyanobacteria [36].

Curiously, the Q cycle is supposed to average ~10 milliseconds. Once again, we have the same conundrum that we had in the mXM (microsomal xenobiotic metabolism) and mOxPhos mechanistic puzzle [14, 21]- the individual steps in a sequential scheme seem to need a longer time window than the overall reaction time-frame. Working with photosystems, several investigators have shown that the intermediates resulting out of photoexcitation are stable for several seconds (and this is also evident by the protocols of the Kok-Joliot experiment). The half-

lives of excited states of P700 and P680 differ significantly. Also, the metastable tyrosine radical has a lifetime of several tens of minutes to hours [27]. Tyrosine is supposed to relay an electron to the charge deficient positive species of PS II RC₂. Since RC₂ would have got the electron from tyrosine, it must mean that tyrosine stays as a cation radical regardless of the status of MnComplex. This does not make any functional sense because electron must be discharged from tyrosine only because of a tug from the PS II RC, and it must get replenished immediately from the WSC. Only if this happens, tyrosine could function as a mediator of redox relay. Also, if the redox potential of tyrosine were higher than 815-820 mV, it would sponsor lysis of water on its own merit. The fact that tyrosine radical is stable shows that its factual redox potential is lower than 815-820 mV. Therefore, it is difficult to envisage the functional viability of Z-scheme ETC mechanism and its synchrony with the Kok-Joliot cycle. When the overall ETC has millisecond duration and the electron relay is supposedly set in spontaneously at the charge of the intermediates at the top, the intermediates should not accumulate for such durations. This can only result if the downward ET processes were bottlenecks. If that were so, the operations 1 & 2 cannot be synchronized. Therefore, even temporal factors discount the coherence of Z-scheme & Kok-Joliot cycle. Why did one go through this elaborate presentation of the sequence of operations and states within an idealized chloroplast? It was done to understand/demonstrate the fastidious or improbable nature of the reaction scheme espoused by the prevailing paradigm. From the minimal chloroplast model (Table 2) of the Z-scheme, one can see that the various reaction centers must undergo a precise orchestration of charged and unstable radical species in well defined space-time frames.

With reference to the model/data in Tables 2 & 3, there is no logic available to explain how the system times and synchronizes the overall processes that occur at various locales at vastly different time scales. It can be seen that the timescales of reactions last from a few picoseconds to a few milliseconds. The qualitative probability rating in the last column is derived as follows: If we accept the first two and last two reactions as true experimental values, then all reactions must fall significantly below the millisecond time-scales. Further, only steps 5, 7, 8 and 13 have energy terms to enable a proton pump (which requires ~21 kJ/mol); but most of these steps involving quinones are located at the membrane phase where protons are unavailable (as per CRAS proposal). Perhaps, only step 8 could qualify for this role chemically and energetically,

and that too would give only a single proton equivalent. But when one considers the energy input for upstream pumping and protons needed for PQ recycle (PQH₂ production from PQ) part of the Q-cycle at Cyt. *b₆f*, this energy window also is unviable. This is even with the CRAS-apologists' consensus of ~2.5 protons for one ATP [37]. Therefore, the Z-scheme's prescribed route of electron flow neither explains Emerson's observations [25] nor can it support proton pumps for chemiosmotic ATP synthesis.

For an elementary series reaction of the type $X \rightarrow Y \rightarrow Z$ with rate constants k_X and k_Y respectively, the conversion rates may be expressed in simple first order terms as: $-r_X = k_X * C_X$ and $-r_Y = k_Y * C_Y$. Then, with basic mass action and calculus treatments, if C_{X0} is the initial concentration of X, we have at time t:

$$C_X = C_{X0} * e^{(-k_X.t)}$$

$$C_Y = (k_X * C_{X0}) * [e^{(-k_X.t)} - e^{(-k_Y.t)}] / (k_Y - k_X)$$

$$C_Z = (C_{X0} - C_Y - C_X) = C_{X0} [k_Y * (1 - e^{(-k_X.t)}) - k_X * (1 - e^{(-k_Y.t)})] / (k_Y - k_X)$$

With the above concepts, let us attempt to study the sequential reaction within three pit-steps of the Z-scheme, say: PC^+ to PC to PS I (and thereafter to Fd and NADPH). [For citations of sources of constants, please refer our earlier work [38]. That is, the multi-molecular or nth-order reactions are minimally represented in first order as:

Since the Z-scheme is serially connected from water to $NADP^+$, the concentration of electron relay catalysts end up being the actual concentration terms of reactants. The starting concentration of PC is ~1 μM ; and PS I is either equal or sub-stoichiometric to this amount [39]. Therefore, owing to the laws of mass action, the amount of products at any time 't' cannot exceed the limiting concentration of an electron conveyer like PC, which is in low micromolar levels. Concentration of NADP in chloroplasts is ~500 μM during the light reaction and the purported dissociation constant of oxidized and reduced FNR for $NADP^+$ is 14 μM and ~1 μM

respectively (i.e. 10^{-6} to 10^{-5} M), as detected by spectrophotometry and the midpoint potential of FNR is 377 mV and NADP^+ is 320 mV (both values are for the two electron reaction) [40]. Quite simply, the serial connectivity and the thermodynamic drive are inadequate to explain the physiological or equilibrium concentrations observed in chloroplasts. In a typical chloroplast, there are $\sim 10^2$ thylakoids, with each thylakoid containing $10^2 - 10^3$ PS I / PS II and there are approximately 10 folds the quinones per a given PS. Now, data from a meticulous study [39] provides crucial information for understanding the plastocyanin distribution in chloroplasts. They found that- (i) in dark, plastocyanin is relatively more abundant in stroma, than in grana, (ii) ~ 3 PC molecules were found per every couple of micrometers of lumen, (iii) total count of PC varied from 10^2 to 10^3 per chloroplast. This corresponds to a 1:1 stoichiometry of PS I and PC. The diffusion constant of PC is ~ 10 - $100 \mu\text{m}^2/\text{s}$ [41] which implies that an average PC molecule can muster a displacement rate of 3-10 nm/ms. Such dimensions are in the approximate range of the linear spread of a PS system and the width of lumen or membrane. Clearly, the availability and mobility of PC would be a constraint for a millisecond time-frame reaction. Let's go to an earlier step. The electrons have to reach from the deep-seated appressed granal PS II to Cyt *b₆f* via PQ to PC. If we see the experimentally calculated PQ's diffusion coefficient of 0.01- $0.3 \mu\text{m}^2/\text{s}$ within thylakoid membranes [42, 43], the maximal linear displacement rate is only 0.2 Å/ms. This means that PQ is practically immobile with respect to the limiting reaction timescales. Considering the high density of protein packing in the thylakoid membranes, the diffusion would only be more torturous and the lateral displacement rate would only be lower than the ones calculated. Though the complete reduction of PS I only takes a few decades of microseconds (and thus, is not rate-limiting), the electron translocation afforded by PQ and PC would surely become rate-limiting. Therefore, the synchronization of Operation 1 & Operation 2 cannot be achieved in the deterministic way (as espoused by Z-scheme and Kok-Joliot cycle), and consequently, the elaborate Z-scheme ETC for P1-Pp is untenable owing to the existence of multiple rate-limiting steps. [[Warburg had originally observed that a given amount of light reduces more CO_2 when allowed to fall on the cells intermittently, rather than continuously. When a frequency of 133 flashes of light were employed per second with equal time periods for light and dark phases, almost a 100% enhancement in photosynthetic yield could be noted (when compared with the provision of light in a continuous mode). The yield (oxygen produced) was enhanced to 400% by Emerson, using 50 flashes per second, but with longer dark periods. This

means that light was flashed for 3.4 ms followed by a dark phase of 16.6 ms. Therefore, the slower reaction must be the recharging step of the RC. These data and facts only confirm the analyses made in the earlier paragraphs.]]

7. Can the Q cycle explain quinone recycle and can Kok-Joliot cycle explain oxygenesis? A Q-cycle comprises of two parts and in each part, a fresh molecule of reduced plastoquinone (QH₂) binds to a given locus of the Cyt. *b₆f* trans-membrane region. Throughout the two parts of the Q-cycle, one molecule of oxidized quinone binds to another locus of the trans-membrane side of Cyt. *b₆f*. In each part of the Q-cycle, one PC molecule binds to the lumen extension of Cyt. *b₆f*. In Table 3, only the reduction of PC was highlighted in the scheme was shown. The other part, of reducing plastoquinone by using the electrons from plastoquinol, is one of the most “incredible” reaction mechanisms proposed in biochemistry. It advocates that electrons must go downhill and uphill in the same circuit. Quite strangely, heme x has a redox potential of 100 mV, which would enable it to receive electrons from PQH₂ [44]. But thereafter, the redox potentials of the remaining part of the circuitry could not be traced from literature, to enable a return of the electrons to PQ, which has a redox potential of 80 mV. (This part of the cycle cannot have only downhill connectivity and considering the distances involved, the recycling would be very inefficient.) Therefore, this multi-molecular scheme would be highly disadvantaged with respect to thermodynamic gradients, mobilities of the concerned components and seems to be highly improbable. Surely, it is difficult to reason how or why a molecule would go through a redox cycle to generate itself. This would mean that electron transfer would have little little energy loss in the process, which would be against fundamental laws of thermodynamics. Our critical dissection of the Q-cycle in the mitochondrial Cyt. *bc₁* complex is also worth mentioning here [16, 17, 20, 21] and almost all the arguments used there are applicable in the current context too.

One can speculate intuitively and logically that it would be a low probability event for the WSC to hold on to two water molecules and their reaction intermediates for an extended time period, ranging into milliseconds. Such physiologically improbable paradigm was also accepted in the mXM and mOxPhos community too, but our explanations provided better mechanistic routes [15-17, 45]. Further, if oxygen were to be involved in electron transfers, then we would expect a consumption of the species within an oxygen limited environment (such as the Kok-Joliot’s

setups of the first few cycles). Also, high partial pressure of oxygen does not lower rate of oxygen evolution [46]. But photosynthesis is lowered at high light intensity and oxygen concentration (the Warburg effect (reference is needed here)). This is an outcome that cannot be explained by the prevailing concepts. Therefore, it is imperative that we go beyond the Kok-Joliot model of oxygenesis, particularly under the light of Emerson's 'sidetracked' observation that illuminating PS I alone also gave oxygenesis [26]. We have presented a more detailed critique of the assumption and observations that led to the formulation of Kok-Joliot cycle in a recent article elsewhere [25].

8. *Can the Z-scheme explain photophosphorylation?* Z-scheme necessitates an automated and deterministic movement of electrons through several species, releasing oxygen and NADPH; and does not directly couple the redox process with ATP synthesis. (That is- the CRAS-ZS model is an electro-mechanical 'phospho-ester bond making' proposal.) The charge separation scheme of K-J cycle at Mn-Complex of PS II dictates the formation of protons within the inner thylakoids of grana whereas ATPases are located at the peripheral thylakoids or stromal lamellae. This predicament does not afford a viable proton cycling strategy for the closed-pot Mitchellian CRAS. Further, we have argued earlier that the CRAS scheme fails to explain physiological ATP synthesis, owing to a multitude of kinetic and thermodynamic issues, [16, 17, 20, 25]

9. *Does the Z-scheme agree with evolutionary principles?* The Z-scheme and CRAS mechanism is quite unlike the other accepted biocatalytic mechanisms. Any one of the components of chloroplasts cannot serve an independent function without the presence of a numerous number of other components, in the Z scheme purview. This makes the Z-scheme and CRAS mechanism a typical example of "irreducibly complex" (Michael Behe's writings), a catch 22 in evolutionary logic. Given the multitudes of queries and untenable suppositions exposed, it is unlikely that a mechanism like the Z-scheme could have evolved from a minimal set of components. Particularly, when there is ample oxygen/DROS (in steady state) to shunt all the deterministic machineries needed for its functioning, it is difficult to envisage how the non-specific Z-scheme ETC could function so deterministically, against all odds. Further, the Z-scheme does not afford

any tangible evolutionary rationale as why leaves that exhibit high photosynthetic rates die sooner [47].

10. Can the Z-scheme explain the presence of ROS in chloroplasts? We had pointed out that traditional bioenergetic perspectives were unfeasible because they discounted - (i) the catalytic roles of ROS and their ubiquitous formation in redox phosphorylations & (ii) the energy drive afforded by extra-organellar protons in the dynamic process [16, 17, 20, 21, 25]. One of the most salient features of chloroplast physiology is its ability to generate ROS (and ROS's positive correlation to ATP synthesis) and chloroplast's ability to facilitate ATP synthesis when presented with low extra-organellar pH (as seen in the famed Jagendorf experiment). Both of the above aspects downplay the CRAS-ZS model, which is at a loss to explain- (i) the vast amounts of one-electron redox-active species randomly distributed across the thylakoids that has high propensity to form ROS, and (ii) how a system that requires a “closed pot for recycling protons” is facilitated by high amounts of extra-organellar protons. We have recently explained such outcomes with the murburn explanation, which is well-grounded in thermodynamics, kinetics and probability [25].

Consolidated criticism of Z-scheme model of Pl-Pp (integrating/complementing earlier discussion)

As per the extant paradigm [8], PS II serves to trap light energy and Cyt. *b₆f* helps to recycle the quinols formed at PS II; and both proteins ‘pump protons’ to generate a proton-gradient across the thylakoid membrane. The nanoscale dimensions of thylakoids and the alkaline pH value of 7.8 - 8.2 [48, 49] discount the viability of proton pumps at resting state [21, 22]. In such a scenario, an additional ~100 kJ/mol would be required [~79 kJ/mol for heterolytic dissociation of water ([50] 33) and 21 kJ/mol for the process of pumping out protons to generate *pmf* [8, 21]. Since the quinone (0.1 V) to plastocyanin (0.37 V) redox gradient gives inadequate $\Delta G (= -nF\Delta E = \sim -104 \text{ kJ/mol}$ for a 4e reaction), the CRAS proposal becomes energetically nonviable. Further, the Z-scheme does not offer reasons why- (i) ~5 nm lumen-ward extensions are found in PS II [51] and Cyt. *b₆f* [52], (ii) certain redox centers within the ETC are located far apart with little or unfavorable redox gradients, (iii) the numerous “non-route” redox-active pigments

located in the membrane or DRS do not shunt the purported electron-circuitry, (iv) redox transfers are made by taking/giving one or two or four electrons, and (v) the electron transfers are spatially demarcated and span across three phases. For example- the current awareness on PS II structure reveals that while the reaction center of PS II is embedded in the trans-membrane region, the MnComplex is located in the lumen side, >2 nm away [53]. After photo-electronic discharge, it is unknown how the reaction center would command a suitable response from the MnComplex. Or, since the MnComplex is not photo-active on its own merit, there is no known logic for a spontaneous one-electron supply from the source of “2H₂O-MnComplex” via a four-electron KJC. It is also unforeseeable how the MnComplex could hold on to the various species formed by electron/proton or hydrogen atom abstraction from two water molecules. This predicament is quite similar to the mechanistic perception of mitochondrial cytochrome oxidase holding on to a molecule of oxygen, waiting for four protons and four electrons, to produce two molecules of water and “pumping protons” in the redox process [21]. From the redrawn Z-scheme in Figure 3, the chinks in electron transfer are clearly evident. PS I or II can only accept electrons when they are positively charged (a highly reactive transient phase) and it is not clear how the electron supply is deterministically timed in these systems. The bulky semiquinones and quinols (PQ^{*-} and PQH₂) would have low mobilities within the lipid phase owing to their phenyl head’s polarity, extra bulk/length and isoprenoid nature of the tail. The tetra-molecular Q-cycle is a low-probability scheme that seeks a millisecond-scale binding of three different molecular species (quinone, quinol and oxidized plastocyanin) to three distinct loci of Cyt. *b₆f*. In vitro, the experimentally determined plastocyanin(PC)-Cyt. *b₆f* interaction presents a K_d of ~10⁻⁴ M (two orders higher than the physiological PC concentration of 10⁻⁶ M!), and the equilibrium constant (K_{eq}) for PC’s reaction with Cyt. *b₆f* is only 2 x 10⁰ [54]. This is very low compared to the 1e or 2e bimolecular murburn reactions of semiquinone+DROS with K_{eq} values of 10¹⁵ to 10³¹ and/or K_{eq} values of 10¹⁰ to 10²⁶ for recycling of quinol with oxygen [as shown in Table 2 of [20]. Rate constant (*k*) values of 10⁷ to 10⁸ M⁻¹s⁻¹ were noted [54] for the oxidation of Cyt. *b₆f* and reduction of PS I by the mobile Cu-protein. Since PC is available at ~10⁻⁶ M in chloroplasts, levying a limiting substrate constraint of 10⁻⁶ M gives a timescale of 10⁻¹ to 10⁻² s for PC mediated reactions at Cyt. *b₆f* and PS I, which cannot be reconciled with- (i) the 10⁻¹² s timescales for excitation and electron-discharge of the two reaction centers in the middle of the sequence, (ii) reactions of 10⁻⁶ and 10⁻⁹ s timescales at several pitstops/junctions, and (iii) oxygen

liberation at the beginning or NADP reduction at the end of the sequence, both of which occur at $\sim 10^{-3}$ s timescales. If we consider that physiological concentration of protons is $\sim 10^{-8}$ M in chloroplasts (making it practically unavailable in a resting thylakoid), Cyt. *b₆f* mediated Q-cycle and proton-pumping become an “improbable” component relative to the millisecond timescale experimentally known for product formation. Therefore, the serial linking of Cyt. *b₆f* with PS II and PS I does not afford temporal viability. Q-cycle also seeks a thermodynamically inexplicable outcome that as the quinol gets oxidized by PC, electrons must also flow from the quinol to quinone within a purported circuitry where redox pitstops are separated by distances >10 Å. Therefore, the proposal that “the relatively bulky plastoquinone (PQ, 0.75 KD) undertakes a deterministic journey in the membrane phase from PS II located at deep loci within the grana to Cyt. *b₆f* located in peripheral thylakoids, and PC (~11 KD) relays the electron from thereon via the aqueous phase to PS I” makes little kinetic logic. Across the known structures, the minimal Cu-Fe distance in PC-Cyt. *f* complex would be >1 nm and Cu-Mg in RC donor chlorophyll distance in PC-PS I complex would be >2 nm. Conservation of a small hydrophobic patch and approximate redox potential in PC [53] cannot overcome the fact that surface residues and Cu-coordination sphere vary significantly across various species. This is not expected ‘evolutionarily’ if PC served as the protein-protein electron transfer liaison between the photosystems. Most importantly, as demonstrated for the mitochondrial Q-cycle at Complex III or Cyt. *bc₁* [20], the deterministic Q-cycle at thylakoids’ Cyt. *b₆f* would not afford adequate energetic yield to enable proton-pumping and ATP-synthesis.

Analyzing from a reaction energetics perspective, if the photolysis occurred via an overall 2e paradigm of the CRAS-ZS model, the minimal *nett* transformed Gibbs’ free energy that would entail is [17, 20, 55]-

However, the input of 4 einsteins each of 680 nm (175.9 kJ/mol) and 700 nm (170.9 kJ/mol) photons give only 1387.3 kJ/mol. Therefore, the extant paradigm advocates that the plant photosynthetic system works with an impossible efficiency of ~105%, as calculated by reaction energetics. It is to be restated that the energy for water-heterolysis and pumping protons are still unaccounted in the above calculations. Further, simple calculations presented here show that the PS I reaction alone would need ~1946 kJ/mol (because the Cyt. *b₆f* reaction would be ~ -104 kJ/mol, as discussed earlier), corresponding to a minimal requirement of 12 photons. Since the PS II reaction minimally needs 4 photons (for liberating four electrons), Z-scheme would necessitate that PS I should far exceed PS II by several folds within the chloroplasts, whereas the reality is that PS II is usually found at higher concentration than PS I. Summating, the following quantitative facts remain at odds with the extant explanations: (i) By virtue of being microdimensioned organelles, mitochondria and chloroplasts are practically “aprotic” systems that have only ~10¹ ‘free protons’ (which cannot cater to ~10⁵ redox proteins!). Therefore, it is a quantitatively untenable concept that proteins pump protons in such organelles [16, 17, 19, 21, 22]. (ii) Several steps within the purported scheme (electron transfer coupled to proton pump) cannot occur with kinetic and thermodynamic viability via affinity-driven binding and Marcus outer sphere mechanism [21, 56]. (iii) The Complex V sponsored equilibrium driven “electromechanical” ATP synthesis is a low probability event that could only give picomolar levels of ATP in physiological realms [16, 17]. Furthermore, the lack of adequate explanations for inhibitions and the recent elucidation of a murrumbidgee-based mechanism of CN-toxicity in various redox systems [13, 15, 18, 45, 57, 58] warrants a revisit of the explanations for PI-Pp. Since the overtly deterministic Z-scheme-KJC paradigm has kinetically incompatible elements, overlooks key observations for photosystem/pigment dependent trace of oxygen transients [9, 10], cannot explain the distribution of protein complexes or structure-function correlations of PS II/Cyt. *b₆f*, and violates fundamental laws of thermodynamics, we deem it imperative that the founding assumptions and equations for PI-Pp are revamped.

Avenues for better explanations and further explorations

Historically, several stalwart scientists like Edward Slater (pioneer in the field and long-term editor of BBA-Bioenergetics), Britton Chance (inventor of stopped-flow enzymology), Robert Williams (Irving-Williams series fame), Walter Wainio (pioneer of cytochrome oxidase structure-function) and Gilbert Ling (inventor of Ling-Gerard electrode) had questioned Peter Mitchell's proposal that "electron transfers in mitochondrial/chloroplast membrane proteins is coupled to phosphorylation of ADP via a *proton motive force (pmf)*" [21]. Daniel Arnon, the renowned plant physiologist (of cyclic/noncyclic photophosphorylation fame) had challenged the Z-scheme with valid arguments/evidence and proposed an independent functioning of PS I & II [56], only be sidelined by entrenched peers [59]. These instances are quoted to demonstrate that Pl-Pp mechanism is a highly daunting conundrum, dogmatic perspectives prevail in the field and a global consensus was never present within the research community (except among the victorious ones of the 'OxPhos' wars). This is because of the complexities/challenges involved in- (i) light's interaction with the numerous pigments arranged in diverse configurations within the intricate chloroplast architecture, (ii) unraveling the logic of energy transduction and electron transfers among the discretely distributed proteins on the thylakoid membrane/lumen and stroma, (iii) pin-pointing the reactions and site(s) of oxygen evolution, and (iv) understanding the coupling of the three processes above to the modality of ATP synthesis.

Moving on, a new explanation is available in murburn concept (from "*mured burning*", implying closed oxidation). It is an evidence-based redox mechanistic paradigm that professes ubiquitous/stochastic interactive redox equilibriums among molecules, unbound ions and radicals [12]. Murburn schemes advocate that diffusible reactive (oxygen) species (DRS/DROS/ROS; exemplified by O_2^{*-}/OH^- ions, $H_2O_2/^1O_2$ molecules, *OH radical, e_{aq} , etc.) serve as crucial elements in metabolic electron/moiety transfer processes [12]. We used the insightful structural information on membrane redox proteins to advocate a murburn paradigm in heme/flavin enzymology [60-62], xenobiotic metabolism (mXM) [14], unusual physiological dose responses [63], mitochondrial oxidative phosphorylation (mOxPhos) and thermogenesis [16, 17, 19, 22]. The acclaimed CRAS paradigm of both mOxPhos and Pl-Pp encompasses electron transport chain (ETC) proteins serving as proton pumps to generate *pmf*, which is harnessed by a rotary ATP synthase function [8]. The mOxPhos toxin of cyanide (CN) is also an established inhibitor of Pl-Pp system and its components [64-67]. Another mechanistic commonality is that

uncouplers (substituted phenolics like dinitrophenol) disrupt ATP synthesis in both the membrane redox systems [8]. In this regard, we pointed out that substituted phenolics critically affect electron transfers even in mXM and since this system does not involve any “harnessing of *pmf*”, such uncouplers are more likely to serve as interfacial proton/DROS modulators (and not trans-membrane proton-shuttlers) [13, 16, 17, 19, 22, 63, 68, 69]. The commonality in the nature of catalysts (porphyrin/flavin/FeS-bound proteins working at phospholipid interfaces), cofactors (nicotinamide nucleotides), and products (DROS+Heat+H₂O/O₂) is also evident across the systems. The molecular species of oxygen, DROS and water are also ubiquitous in the microsomal, mitochondrial and thylakoid systems, and their roles cannot be restricted to any unique locus, per se. Most importantly, these membrane-interface reaction systems show non-integral and variable stoichiometry, and a ‘thermodynamic pull’ exerted by the presence of ‘substrates/uncouplers’ [13, 15-17, 19, 22, 63, 68, 69]. These similarities and parallels in the phenomenology of mXM, mOxPhos and Pl-Pp systems (Table 1) argue for “*a stochastic paradigm involving DROS as delocalized operative/transducing elements*” in the three redox routines. Recent theoretical and experimental (in vitro, and in situ) demonstrations of DROS-mediated electron/moiety transfers in xenobiotic hydroxylation, ADP phosphorylation, etc. [15-17, 19, 22, 63, 68] consolidate the murburn purview.

Conclusions

With the ten critical queries posed above, the Z-scheme’s inadequacies are amply demonstrated. These analyses justify Daniel Arnon’s (a pioneer plant physiologist from Caltech., who proposed the cyclic and non-cyclic mode of photosynthesis) questioning of Z-scheme [56]; which was unfortunately unheeded by the peers of his times [59]. We have shown herein that it is quite a circuitous and low-probability route that an electron derived from water must go through the elaborate path of Kok-Joliot cycle, P680 cycle, plastoquinone relay, Q-cycle, plastocyanin relay, P700 cycle, ferredoxin relay and reach its sink at flavoreductase, in order to make NADPH. Physically, the four pit-stops of chloroplast Z-scheme ETC (PS II, Cyt. *b₆f*, PS I and FNR) are purportedly inter-connected via three mobile electron relay agents (PQ, PC and Fd, respectively). Regarding the role of quinones, we have now successfully challenged the Q-cycle paradigm in chloroplasts and proposed a murburn model in lieu [70]. Therefore, the electron relay by PQ

between PS II and Cyt. *b₆f* becomes unviable. Further, it is known that plants and cyanobacteria can survive and photosynthesize without PC/Cyt. *c₆* [71, 72], thereby delinking the other two connections also. Researchers have continued to find several alternative pathways for electron transfers in chloroplasts [73], making Z-scheme more a matter of “tradition”, than a relevant aspect of physiology. Furthermore, we have recently shown via multiple theoretical analyses and experimental observations (as reported by other groups) that PC and Fd are more likely to don the optional roles of redox capacitors, as professed by the murburn model [28]. Therefore, it is opportune that researchers look for alternative explanations for the electron transfer process and re-address the overall phenomenology of light reaction of photosynthesis. In this regard, murburn concept [14, 16, 17] could potentially present a viable alternative worth exploring. In the chloroplast system, the new perspective affords holistic thermodynamic grounding and tangible roles for thylakoid membranes, light harvesting complexes and various pigments [25, 70, 74-76]. We hope this critical article serves the imminent mandate to move beyond the Z-scheme.

Acknowledgments and declarations: The work was powered by Satyamjayatu: The Science & Ethics Foundation. The authors have no conflicts of interests to declare. KMM wrote the first draft of the paper and the other authors provided various inputs/discussions and contributions like drawing figures and presenting better-articulated and substantiated arguments.

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