Biophysical Chemistry 230 (2017) 45–52

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: www.elsevier.com/locate/biophyschem

Two-ion theory of energy coupling in ATP synthesis rectifies a fundamental flaw in the governing equations of the chemiosmotic theory

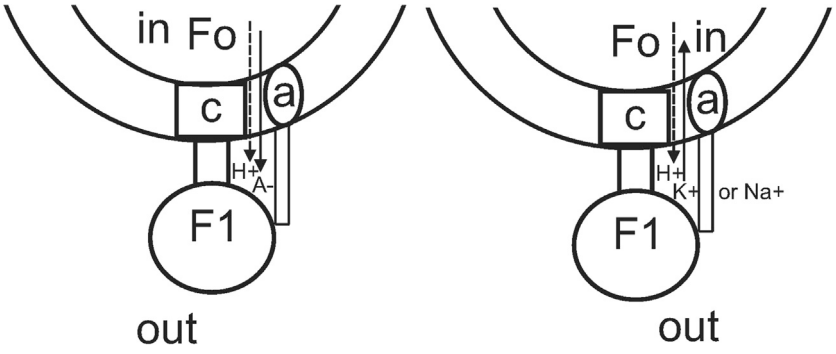
Sunil Nath

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110016, India

HIGHLIGHTS

- A fundamental flaw is identified in the equations of Mitchell's chemiosmotic theory
- The flaw weakens the theoretical foundations of the chemiosmotic theory
- The proposed Nath's torsional mechanism of energy transduction and ATP synthesis eliminates the flaw
- The governing equations of the new theory accurately predicts experimental data
- The two-ion model offers a superior description of energy coupling and ATP synthesis

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Bioenergetics
Oxidative phosphorylation
Photosynthesis
Mitchell's chemiosmotic theory
Protonmotive force
Delocalized electrical potential
Two-ion theory of energy coupling
Nath's torsional mechanism of energy transduction and ATP synthesis

ABSTRACT

The vital coupled processes of oxidative phosphorylation and photosynthetic phosphorylation synthesize molecules of adenosine-5′-triphosphate (ATP), the universal biological energy currency, and sustain all life on our planet. The chemiosmotic theory of energy coupling in oxidative and photophosphorylation was proposed by Mitchell > 50 years ago. It has had a contentious history, with part of the accumulated body of experimental evidence supporting it, and part of it in conflict with the theory. Although the theory was strongly criticized by many prominent scientists, the controversy has never been resolved. Here, the mathematical steps of Mitchell's original derivation leading to the principal equation of the chemiosmotic theory are scrutinized, and a fundamental flaw in them has been identified. Surprisingly, this flaw had not been detected earlier. Discovery of such a defect negates, or at least considerably weakens, the theoretical foundations on which the chemiosmotic theory is based. Ad hoc or simplistic ways to remedy this defect are shown to be scientifically unproductive and sterile. A novel two-ion theory of biological energy coupling salvages the situation by rectifying the fundamental flaw in the chemiosmotic theory, and the governing equations of the new theory have been shown to accurately quantify and predict extensive recent experimental data on ATP synthesis by F_1F_O-ATP synthase without using adjustable parameters. Some major biological implications arising from the new thinking are discussed. The principles of energy transduction and coupling proposed in the new paradigm are shown to be of a very general and universal nature. It is concluded that the timely availability after a 25-year research struggle of Nath's torsional mechanism of energy transduction and ATP synthesis is a rational alternative that has the power to solve the problems arising from the past, and also meet present and future challenges in this important interdisciplinary field of research.

E-mail addresses: sunath@dbeb.iitd.ac.in, sunil_nath_iit@yahoo.com.

http://dx.doi.org/10.1016/j.bpc.2017.08.005
Received 11 July 2017; Received in revised form 8 August 2017; Accepted 17 August 2017
Available online 19 August 2017

1. Introduction

The molecular mechanism by which energy is released, conserved, transduced, stored, and utilized in biological processes and how chemical reactions can be coupled are among the most fundamental questions in science. The prevailing theory of energy coupling serves as a guide in several fields of biochemical research. In many fields of biology, in the eloquent words of Green [7], "The whole direction of research is profoundly influenced by the prevailing theory of energy coupling. It plays a comparable role in bioenergetics, as does atomic theory in physics or valence theory in chemistry." Almost all the biochemistry textbooks currently in print use the chemiosmotic theory of energy coupling to explain the fundamental processes of oxidative phosphorylation (OX PHOS) and photophosphorylation.

In what has been termed as "the OX PHOS wars," a number of stalwarts of the latter half of the 20th century – E. C. Slater, R. J. P. Williams, Albert Lehninger, David Green, Gregorio Weber, among others – had expressed their very strong objections regarding the correctness and validity of the chemiosmotic theory [7,27,29,34,35], and the theory had clearly been shown to be inconsistent with a considerable body of accumulated experimental evidence, summarized recently [11,20,23,37]. Yet, the scientific ingenuity of these and other giants of biochemistry and biophysics could not formulate a theory that might be put in place of chemiosmosis, despite several attempts, and the plethora of experiments failed to offer any positive guidance to devise a rational substitute. Perhaps partly as a result of this failure, textbooks continued to cause confusion in young and old minds alike.

The author's research of the past twenty-five years had consummated a superior and more detailed alternative molecular mechanism – the Nath's torsional mechanism of energy transduction and ATP synthesis – that went beyond the chemiosmotic theory (for reviews see [1,2,8,11,19–21,24,26,33,37,38]). The chemiosmotic theory postulated a role solely for proton translocation through the ATP synthase, and violated overall electrical neutrality of bulk aqueous phases [24]. On the other hand, the new paradigm of the torsional mechanism proposed that the ATP synthase is a *cotransporter*, contradicting a fundamental postulate of chemiosmosis. The torsional mechanism was quantitative and contained mathematical equations that described the overall driving force of ATP synthesis arising from discrete proton and *anion/countercation* translocations in the membrane-bound F_O portion of F_1F_O-ATP synthase. These translocations occurred by a dynamically electrogenic but overall electroneutral mode of ion transport in the F_O portion of the ATP synthase that did not violate overall electroneutrality [21,24]. Which of these two contrasting models offers a better approximation of the real process of biological energy coupling in OX PHOS? If one of these models was false, then where exactly did the error lie? What was its exact origin? Such an analysis would also help in the avoidance of costly and debilitating errors in the future in various connected and interdisciplinary fields.

2. Mathematical analysis of the governing equations of the chemiosmotic theory

It appeared to this author that the governing mathematical equations of the chemiosmotic framework had not been scrutinized earlier, despite the passage of over fifty years. Textbook versions of the chemiosmotic theory of energy coupling are based on Mitchell's classical paper [14], one that has also been reproduced in recent compilations. The governing equations of the theory were given therein as Eqs. (7)–(15) [14], and the concept of the "protonmotive force" (Eq. (15)) was also introduced for the first time in that paper. Eqs. (1)–(6) in that work refers only to illustrative diagrams and to certain hypothetical schemes of coupling in OX PHOS, so Eqs. (7)–(15) forms the conceptual underpinning of the theory.

Interestingly, in the original, more primitive form of the theory of chemiosmotic coupling, Mitchell only postulated a "proton-impermeable membrane" with ΔpH as the sole driving force of ATP synthesis in the OX PHOS process [13]. Unfortunately, the ΔpH in mitochondria was found to measure less than one unit, and therefore insufficient in magnitude to act as the driving force of ATP synthesis. Hence Mitchell was forced to modify (or in his words, "sophisticate") his theory by postulating that the coupling membrane has a low permeability to *all* anions and cations generally (and not just to H^+), thereby allowing a *delocalized* electrical potential (given the symbol ΔE in his 1966 paper) to be conserved across the membrane and provide the *major* part of the driving protonmotive force, Δp, with need for only a relatively small pH difference, ΔpH.

Moreover, as discussed in Section 1, the author's research of the past twenty-five years had consummated a detailed alternative molecular mechanism that went beyond the chemiosmotic theory [19–21], and he could predict with complete confidence that the 1966 "derivation must be erroneous," and that the error must lie somewhere between Eq. (9) (which was correct, under the assumptions made) and Eq. (15) of Mitchell [14] containing the delocalized potential, which was unsupported by theory (Nath, S., personal communication to E. C Slater, 2010 [22].).

Assuming a treatment based on equilibrium thermodynamics following Mitchell [14], the overall ATPase reaction was represented by.

$$ATP + H_2O + nH^+{}_R \leftrightarrow ADP + P_i + nH^+{}_L \quad (1)$$

where L and R stand for bulk aqueous phases on the left and right of the energy-transducing membrane, and n is the number of H^+ ions coupling oxidation and phosphorylation.

With activities enclosed within curly brackets, the normal hydrolysis equilibrium for ATP can be written as

$$\frac{\{ADP\} \times \{P_i\}}{\{ATP\}} = K'\{H_2O\}\frac{\{H^+\}_R^n}{\{H^+\}_L^n} \quad (2)$$

According to the chemiosmotic theory, the value of n in Eq. (1) and Eq. (2) equals two [16] [a number that Mitchell vehemently asserted until his dying day, even in the face of adverse experimental evidence that unequivocally showed significantly higher values of proton stoichiometry per ATP; for a thorough discussion of this point, see Sections 5 and 6.3 of Nath [24]], implying that the reaction is strictly coupled to the translocation of 2 protons from one bulk aqueous phase (R) to the other bulk aqueous phase (L) per ATP, i.e.,

$$\frac{\{ADP\} \times \{P_i\}}{\{ATP\}} = K'\{H_2O\}\frac{\{H^+\}_R^2}{\{H^+\}_L^2} \quad (3)$$

It is assumed in the original analysis that ADP, P_i, and ATP all participate in the equilibrium from the same phase, and therefore the activities can be set approximately equal to the concentrations as and when required [14].

In the presence of a delocalized membrane potential measuring ΔE mV between phases L and R (with phase L taken as positive), Mitchell writes the relationship (Eq. (10) in Mitchell [14])

$$log_{10}\frac{\{H^+\}_L}{\{H^+\}_R} = pH_R - pH_L + \frac{\Delta E}{Z} \quad (4)$$

where Z = 2303 RT/F, and F and R are the Faraday constant and the gas constant respectively. However, from its basic definition, $pH = -\log_{10}\{H^+\}$, and applying the definition to the L and R phases, we have $pH_L = -\log_{10}\{H^+\}_L$, and $pH_R = -\log_{10}\{H^+\}_R$. Combining these equations we obtain as an identity

$$log_{10}\frac{\{H^+\}_L}{\{H^+\}_R} = pH_R - pH_L \quad (5)$$

Hence, in reality, Eq. (5) is the correct equation that should have been employed in place of Eq. (4) written by Mitchell [14] arbitrarily and without logic. Comparison of Eq. (5) with Eq. (4) reveals that ΔE/Z is *identically* zero in Eq. (4), which corresponds to Eq. (10) in Mitchell [14]. In other words, it is not possible to derive an Eq. (4) containing a

finite delocalized electrical potential, and certainly a finite, non-zero ΔE cannot be introduced forcibly into the equations in this way [14].

The above analysis reveals that ΔE is zero in Eq. (4) [Eq. (10) of Mitchell [14]], and a main purpose of the present note is to show this. This fact strikes at the root of the mathematical framework of chemiosmosis developed in Mitchell [14], because once ΔE is always zero, it is not possible to derive any of the subsequent equations [Eqs. (11)–(15) of Mitchell [14]]. Thus, substituting ΔE = 0 in each of Eqs. (11)–(15) in the 1966 work, while retaining the other assumptions made subsequently, i.e., that the concentration of inorganic phosphate equals 10 mM, the temperature is 27 °C, and that the ATP/ADP couple is poised centrally, leads finally to the equation for the protonmotive force (in units of mV)

$$\Delta p = -60\Delta pH \tag{6}$$

However, it is not possible to derive

$$\Delta p = \Delta E - 60\Delta pH \tag{7}$$

which is Mitchell's central protonmotive force equation, Eq. (15) in Mitchell [14]. This crucial matter was discussed with a founding-father of bioenergetics, and proponent of the first theory of oxidative phosphorylation [28] during a 15-year long correspondence. He concurred that, "I do agree with your conclusion that Eq. (10) implies that ΔE is zero" (Slater, E. C., personal communication to S. Nath, 2010) [30].

3. Major biological implications

The analysis in Section 2 has major biological implications. Upon invoking a zero ΔE in Mitchell's Eq. (7), there is no finite contribution to add to the ΔpH term. Hence the Δp will not meet the *unremitting* requirement that it be thermodynamically competent to drive ATP synthesis, and one is back to the problem of an insufficient driving force that had historically forced Mitchell to modify his theory in the first place [14].

The above problem cannot be resolved in ad hoc or simplistic ways. For instance, it is not possible to stipulate that the concept of the electrochemical potential can replace the Mitchell [14] theoretical derivation, because, in the definition of the electrochemical potential, the electrical potential is always *local*, while the chemiosmotic theory postulates *delocalized* electrical potentials across bulk aqueous media created solely by incessant, uncompensated electrogenic translocation of a *single* ion species. Translocation of one proton (or a few protons) will create only a negligible bulk-to-bulk delocalized potential; in fact, it was shown that as many as 80,000 uncompensated H^+ need to be translocated in a single mitochondrion to create the 240 mV delocalized potential required by the chemiosmotic theory [24], in agreement with previous calculations by Tedeschi [32]. To avoid confusion, concepts and mechanisms in competing theories need to be differentiated. For this reason, it was suggested that the local electrical potentials postulated in the torsional mechanism be denoted by a different symbol (e.g., Δψ) from the presumed large delocalized electrical potential differences presumed to exist across bulk aqueous media by the chemiosmotic theory (e.g., ΔE or Δφ) [20].

According to Nath's torsional mechanism of energy transduction and ATP synthesis, the mandate of electroneutrality is extremely stringent. This is exactly opposite to the view of the chemiosmotic theory, according to which "processes involving the transfer of a single ionic species between one phase and another at different electrical potential are permissible and have the same thermodynamic status as processes involving the transfer of a single uncharged molecular species" [15]. In the process of chemiosmosis, bulk electroneutrality can be violated at will, if Mitchell's faulty and completely misleading analysis (that can be shown to violate Gauss's law) in a section entitled, "The Fiction of Electrical Neutrality" ([15], pp. 177–179) is to be believed. In the new paradigm of the torsional mechanism, charge imbalance can be created, and is indeed necessary, but it cannot be *sustained* for long; dynamically, the primary translocation creates a local Δψ that favors the translocation of a counterion (that destroys the local potential) over the translocation of another coion (that would have built-up and accumulated the electrical potential) because of the strict requirement imposed by the principle of electroneutrality. These physical principles are of a very general and universal nature, because the presence of a resisting local Δψ will *prevent* continued electrogenic translocation of that ionic species. Hence, a delocalized Δp made up of a large delocalized electrical potential component, ΔE across bulk aqueous phases, that is created and accumulated by purely electrogenic transport of a single ionic species simply does not exist. Further, since the Δψ is local in the access channels of F_O, but the ΔpH is delocalized, it is incorrect and meaningless to combine and add ΔpH and Δψ in a modified definition of Δp. Moreover, a delocalized and a localized driving force cannot compensate for each other and have to be kinetically non-equivalent. Hence, Δp is a mathematically defined phantom quantity that does not have any physical meaning.

The concept of the electrochemical potential (described for example by Eqs. (8)–(11) in Section 4) is a cornerstone of biophysical chemistry to quantify the energetics of ions and charged species. In this concept, the electrical potential, ψ is a *local* potential, one that resides on that particular species. Hence the property can be ascribed as belonging to a single ion. Its energy is available even in elementary single binding/unbinding and translocation steps *within the membrane* and a change in energetics can manifest itself via the process of ion-protein interactions. On the other hand, the electrical potential ΔE in the definition of Δp in Mitchell's "protonmotive" force equation [Eq. (7)] is a general bulk-to-bulk *delocalized* potential across aqueous media that has no relationship with the local electrical potential. Translocation of a single ion will create only a negligible delocalized potential. Such a chemiosmotic mechanism that is central to the creation of the protonmotive force, Δp in Eq. (7) therefore requires that the translocation of an ion species be electrogenic and that such incessant translocation (uncompensated by movement of any anion in the same direction or countercation in the opposite direction during the entire process) of a very large number of ions across the membrane continuously add to the field until a finite delocalized potential of the required magnitude (~200 mV) is established across the membrane between the two bulk aqueous phases.

Hence, one of the reviewers is right in saying that "Δp is the molar free energy difference of protons across both sides of the membrane" *only if such a purely electrogenic mode of translocation of protons is permissible and operates in the system.* Central to the approach of the chemiosmotic theory is the fundamental assumption of an electrogenic process, which in reality should have been proved, and not assumed. As discussed above and in Section 4, this fundamental assumption is false. In fact, the translocation proceeds by an ordered and sequential step-by-step dynamically electrogenic but overall electroneutral mechanism involving two permeant ions in which the departure from electroneutrality is minimal and local. Electrical imbalance caused by the binding/unbinding of ions/charged substrate molecules in the hydrophobic core of the membrane is extremely unfavorable, and would incur a huge energy penalty. Lack of compensation by secondary H^+ cotransport/countertransport events of such severe charge imbalances caused at/in the vicinity of the binding sites by primary elementary events would have deleterious effects on the structural integrity of the binding sites, especially in the absence of metal binding. In a structural sense, proton cotransport/countertransport is a consequence of the requirement for maintaining the structural integrity of the anion/countercation binding sites in the access channels of the energy-transducing membrane. Therefore a large delocalized potential created solely by electrogenic translocation of H^+ ions cannot be built up in the first place because such an electrogenic mode of ion transport [especially one that violates bulk electroneutrality to an unprecedented extent [24]] that was presumed to exist by the chemiosmotic theory simply does not occur in natural systems, and a reorientation of how we think about the fundamental process of energy coupling is necessary.

Another great danger is to believe that no theoretical foundation of the type attempted by Mitchell [14] is needed (or what is worse – an insidious possibility that must be preempted – that the principal result involving the electrogenic protonmotive force, Δp [Eq. (15) of Mitchell [14], which is Eq. (7) here] is correct even if the original derivation is false), or that experiments alone can help resolve the problems. However, as more and more data has continued to accumulate, the fundamental questions have remained unanswered, or incompletely answered. Interestingly, a vast body of experimental data, for example on the thermodynamic incompetence of Δp in the alkaliphiles and haloalkaliphiles, on the isolation of uncoupler-resistant mutants of OX PHOS, on lipid solubility determining the potency of uncoupler action ceteris paribus, new 3D tomographic structural studies on mitochondria [5,6,25], and several other longstanding observations (summarized in [20,23,33]) have defied explanation based on the classical chemiosmotic theory. As shown here, the theoretical foundations on which chemiosmosis was derived does not hold, and without a fundamentally new theory in bioenergetics, it is difficult to see how the immense challenges in this interdisciplinary field can be successfully met. The ultimate in foolhardiness, which ought to be prevented for the sake of scientific progress, is probably to continue to believe in an old dogma even though it is inconsistent with a vast amount of experimental data and whose derivation leading to its main equation is defective and fallacious. Fortunately, a novel unified theory of biological energy transduction and ATP synthesis/hydrolysis free from logical inconsistencies that has the power to solve our problems and overcome the challenges of the present and the future is finally available.

4. Rectification of the fundamental flaw by a two-ion theory of energy coupling in ATP synthesis

The mathematical analysis in Section 2 proves that Mitchell's attempt to modify the equations of the chemiosmotic theory in order to address the acknowledged energy deficit in ATP synthesis was infructuous and unsatisfactory, and failed to solve the central problem in ATP mechanism and thermodynamics. The problem of energy shortfall was actively discussed in various papers between 1998 and 2002 during the formulation of Nath's torsional mechanism of energy transduction and ATP synthesis (for a review, see pp. 75–80 of [19]) and after a systematic reappraisal, a very different, alternative approach was adopted to solve the conundrum. It was postulated in the new theory that the remaining part of the energy not supplied by proton translocation has to be provided by an *independent* energy source separate from the H^+ ion. Thus, the missing energy was postulated by the torsional mechanism to be provided by a *second ion*, i.e. by translocation of an anion or countercation (depending on the system) that was proposed to occur at the a-c interface in the membrane-bound F_O portion of ATP synthase. Hence, according to the torsional mechanism, in contradiction to the standard model, the ATP synthase is a *cotransporter* [19,21,23,24], as shown in Fig. 1, drawn for the cases of a monoanion A^-, (Fig. 1a) or a countercation, taken here as K^+ (Fig. 1b), but can also be Na^+ etc. The physiological anion/countercation transported through F_O has been identified in chloroplasts, mitochondria, and bacteria following a ten-year experimental search. The strictly conserved signature sequence across species of the anion-binding site has also been proposed [23,24].

The concepts discussed above make the ATP synthase a cotransporter, and not simply an electrogenic H^+ conductor. Obviously, if both monoanion/countercation and proton are translocated simultaneously in F_O, then no local electrical field will be created (and subsequently destroyed) in the half-access channels of F_O, and the torque measured by superb state-of-the-art single molecule techniques [9,39]) cannot be rationalized. Therefore, in this context, the new paradigm proposed a *dynamically electrogenic but overall electroneutral mode of ion transport*, in which an ordered, sequential translocation of the ions involved takes place (anion/countercation followed by proton through

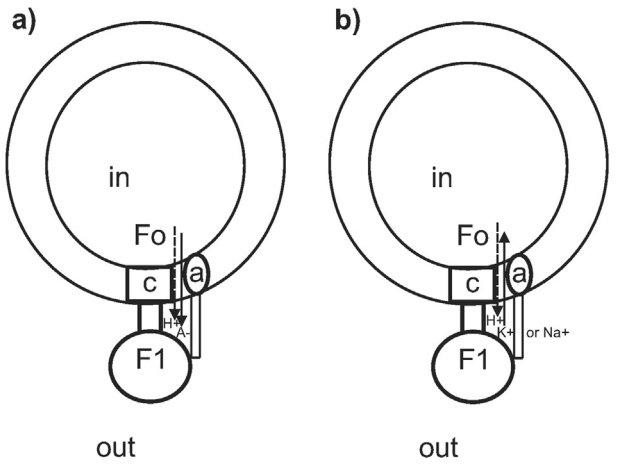


Fig. 1. Two-ion model of energy coupling proposed in Nath's torsional mechanism of energy transduction and ATP synthesis. Bold arrows denote the primary translocation, and dashed arrows the following coupled secondary translocation at the a-c interface in the membrane-bound F_O portion of the F_1F_O-ATP synthase. a) The F_1F_O-ATP synthase as a proton-monoanion cotransporter, or b) as a proton-monocation cotransporter in various organisms.

the F_O access aqueous pathways of the ATP synthase and proton followed by anion/countercation through the redox complexes/photosystems) and a *transient* local field is created and destroyed at/in the vicinity of the ion-binding/transport sites. New terminology of symsequenceport and antisequenceport was coined to describe this unique mode of coupling of proton-anion/countercation flux. Hence the overall driving force for ATP synthesis by the ATP synthase is provided by the electrochemical ion gradients of *two ions*, i.e. protons *and* counterions (anions transported through symsequenceport or cations transported through antisequenceport) and is given by Eq. (5) of Nath [24], which is reproduced here as Eq. (8).

$$d.f. = n(\Delta\tilde{\mu}_H + \Delta\tilde{\mu}_{A/C}) \tag{8}$$

where d.f. denotes the net driving force of ATP synthesis, subscript H denotes H^+, A stands for monoanion, C for countercation, symbol / represents “either or”, and n is the number of discrete translocations of proton and counterion per molecule of ATP synthesized. This equation rectifies the fundamental defect in the chemiosmotic theory discussed in Sections 2 and 3.

The above innovations enabled the torsional mechanism to satisfactorily address the issue of energy shortfall in ATP synthesis (Sections 2, 3), satisfy the necessity of maintaining overall electroneutrality, yet ensure that a dynamic local field is available to generate a mechanical torque in F_O that can be communicated to F_1 and stored (after a cascade of transformations) as torsional energy in the γ-subunit [hence the original, trade-mark name of the mechanism itself [18] and synthesize ATP by a novel catalytic cycle in F_1 using the torsional energy [19]. Hence, in essence, the new theory meets all the varied constraints on the microprocess of ATP synthesis by the ATP synthase, and finally solves the conundrum.

The torsional mechanism satisfactorily addresses the strong criticism levelled by Green against the chemiosmotic theory [7]. In his words, “Energy coupling traditionally has been considered to be a *Ding an sich* and not an extension of classical phenomena. In consequence, the usual rules of chemistry, stoichiometry, and common sense have not been applied to phenomena that were presumed to have their own set of rules. This, of course, opened the door to impermissible liberties with the canons of chemistry, such as the necessity for observing charge neutrality in chemical reactions. The postulate of uncompensated protons moving freely through membranes is one example of such a violation.” These fundamental issues are now convincingly resolved by the new paradigm [37]. It is also of interest to note in this context that in

Table 1
Summary of the calculations for ATP synthesis by thermophilic F_1F_O-ATP synthase reconstituted into proteoliposomes under various experimental conditions.

No.	ADP, mM	Pi, mM	ATP, nM	[ATP]/([ADP][Pi])	pH_{in}	ΔpH	$[K^+]_{out}$, mM	ΔpK	(ΔpH + ΔpK)	Col. 10 for J = 0
1	0.64	10	513	0.08	6.98	1.06	10.9–51.8	0.33–1.01	1.39–2.07	1.76
2	0.64	10	513	0.08	7.62	0.40	51.8–202	1.01–1.61	1.41–2.01	1.70
3	0.32	10	513	0.16	6.98	1.06	10.9–62.9	0.33–1.10	1.39–2.16	1.85
4	0.32	10	513	0.16	7.62	0.40	62.9–202	1.10–1.61	1.50–2.01	1.81
5	0.16	10	513	0.32	6.98	1.06	16.1–76.5	0.51–1.18	1.57–2.24	1.93
6	0.16	10	513	0.32	7.62	0.40	76.5–202	1.18–1.61	1.58–2.01	1.85
7	0.08	10	513	0.64	6.98	1.06	19.6–92.9	0.59–1.27	1.65–2.33	1.98
8	0.04	10	513	1.28	6.98	1.06	28.9–113	0.76–1.36	1.82–2.42	2.12
9	0.02	10	513	2.56	6.98	1.06	28.9–137	0.76–1.44	1.82–2.50	2.19
10	0.64	10	245	0.038	6.98	1.06	10.9–51.8	0.33–1.01	1.39–2.07	1.65
11	0.32	10	245	0.076	6.98	1.06	10.9–51.8	0.33–1.01	1.39–2.07	1.75
12	0.16	10	245	0.152	6.98	1.06	13.2–62.9	0.42–1.10	1.48–2.16	1.80
13	0.16	10	980	0.61	6.98	1.06	19.6–76.5	0.59–1.18	1.65–2.24	2.00
14	0.16	10	2030	1.28	6.98	1.06	28.9–113	0.76–1.36	1.82–2.42	2.14
15	0.32	1	513	1.6	6.98	1.04	28.9–137	0.76–1.44	1.80–2.48	2.15
16	0.16	1	513	3.2	6.98	1.04	28.9–137	0.76–1.44	1.80–2.48	2.18
17	0.08	1	513	6.4	6.98	1.04	42.6–202	0.93–1.61	1.97–2.65	2.30
18	0.08	1	513	6.4	6.37	1.64	10.9–51.8	0.33–1.01	1.97–2.65	2.24
19	0.04	1	513	12.8	6.98	1.04	51.8–202	1.01–1.61	2.05–2.65	2.37
20	0.04	1	513	12.8	6.37	1.64	16.1–62.9	0.51–1.10	2.15–2.74	2.40
20	0.04	1	513	12.8	6.37	1.64	16.1–62.9	0.51–1.10	2.15–2.74	2.40
21	0.02	1	513	25.6	6.98	1.04	62.9–202	1.10–1.61	2.14–2.65	2.47
22	0.08	1	980	12.3	6.98	1.04	51.8–202	1.01–1.61	2.05–2.65	2.35
23	0.08	1	2030	25.3	6.98	1.04	62.9–202	1.10–1.61	2.14–2.65	2.47
24	0.32	0.1	513	15.6	6.37	1.64	16.1–76.5	0.51–1.18	2.15–2.82	2.47
25	0.16	0.1	513	31.2	6.37	1.64	19.6–76.5	0.59–1.18	2.23–2.82	2.52
26	0.08	0.1	513	62.2	6.37	1.64	23.8–113	0.68–1.36	2.32–3.00	2.64
27	0.04	0.1	513	125	6.37	1.64	28.9–137	0.76–1.44	2.40–3.08	2.71
28	0.02	0.1	513	250	6.37	1.64	35.1–167	0.85–1.52	2.49–3.16	2.81

his last published paper [36], R. J. P. Williams sounded the death-knell for chemiosmosis in a final declaration that, “Mitchell's mechanism of ATP synthesis was electrolytic field driven and is impossible” (p. 148 in Williams [36]; see also Nath [24]). He also candidly admitted that, “Both Mitchell and I failed to give a good description of the ATP synthetases” (p. 149 in Williams [36]). Nath's torsional mechanism of energy transduction and ATP synthesis appealed to both founding-fathers of the field, E. C. Slater and Robert J. P. Williams, and succeeded in obtaining their support and stamp of approval in a personal correspondence and exchange of views on ATP mechanism and thermodynamics spread over 15 years.

A simplification of the general Eq. (8) is needed for application to the recent data of Soga et al. [31] for the case of coupled anti-sequenceport translocation of protons and potassium ions where K^+ ions move from “out” to “in” and H^+ ions translocate from “in” to “out” (Fig. 1b). In this case, the net driving force is given by.

$$d.f. = n(\Delta\tilde{\mu}_H + \Delta\tilde{\mu}_K) \tag{9}$$

For the i^{th} ion (either H^+ or K^+ here),

$$\tilde{\mu}_{i,in} = \mu_0' + RTln[i_{in}^+] + z_iF\psi_{in} \tag{10}$$

$$\tilde{\mu}_{i,out} = \mu_0' + RTln[i_{out}^+] + z_iF\psi_{out} \tag{11}$$

where $\mu_0{}'$ is taken as the common standard state chemical potential at the prevailing T, P. Hence, since valence $z_i = +1$,

$$\Delta\tilde{\mu}_H = \tilde{\mu}_{H,in} - \tilde{\mu}_{H,out} = RTln\frac{[H_{in}^+]}{[H_{out}^+]} + F(\psi_{in} - \psi_{out}) \tag{12}$$

$$\Delta\tilde{\mu}_K = \tilde{\mu}_{K,out} - \tilde{\mu}_{K,in} = RTln\frac{[K_{out}^+]}{[K_{in}^+]} + F(\psi_{out} - \psi_{in}) \tag{13}$$

Substituting Eq. (12) and Eq. (13) in Eq. (9), the net driving force of ATP synthesis is given by

$$d.f. = nRT\left(ln\frac{[H_{in}^+]}{[H_{out}^+]} + ln\frac{[K_{out}^+]}{[K_{in}^+]}\right) \tag{14}$$

or,

$$d.f. = 2.303nRT\left(log_{10}\frac{[H_{in}^+]}{[H_{out}^+]} + log_{10}\frac{[K_{out}^+]}{[K_{in}^+]}\right) \tag{15}$$

$$d.f. = 2.303nRT[(pH_{out} - pH_{in}) + (pK_{in} - pK_{out})] \tag{16}$$

and we finally arrive at the simplified equation

$$d.f. = 2.303nRT(\Delta pH + \Delta pK) \tag{17}$$

where $\Delta pH = (pH_{out} - pH_{in})$ and $\Delta pK = (pK_{in} - pK_{out})$ are both positive definite.

Proteoliposomes reconstituted with F_1F_O have been energized by acid-base H^+ transition along with valinomycin-induced diffusion potential of K^+ ions at various ATP, ADP and Pi concentrations and extensive measurements of the initial rates of ATP synthesis/hydrolysis amenable to quantitative analysis have become available only very recently [31]. The results of such kinetic experiments have generally been interpreted in terms of Mitchell's chemiosmotic theory, which is a *single-ion* theory, with its protonmotive driving force, Δp arising solely from H^+ ion translocation, as discussed above. It is shown here by detailed calculation that the results of these kinetic experiments are quantitatively consistent with a *two-ion* theory of energy coupling such as Nath's torsional mechanism of energy transduction and ATP synthesis in which energy for synthesis of ATP is contributed by translocation of two ions, in this case K^+ and H^+. This has major biological implications for models of energy coupling in bioenergetics.

Proteoliposomes reconstituted with thermophilic *Bacillus* F_1F_O (TF_1F_O) were incubated in the acidic medium. The potassium concentration in the acidic medium is taken as $[K^+]_{in}$ and the measured pH of the acidified solution is the pH_{in}. The reaction is initiated by injection of the acidified proteoliposomes into a base medium containing luciferin, luciferase, and valinomycin. The measured potassium and proton concentrations in the resulting solution after mixing correspond to $[K^+]_{out}$ and pH_{out} respectively. By mixing, a pH gradient was established across the membrane which was calculated from the measurements as being equal to (pH_{out} – pH_{in}) = ΔpH. The valinomycin-

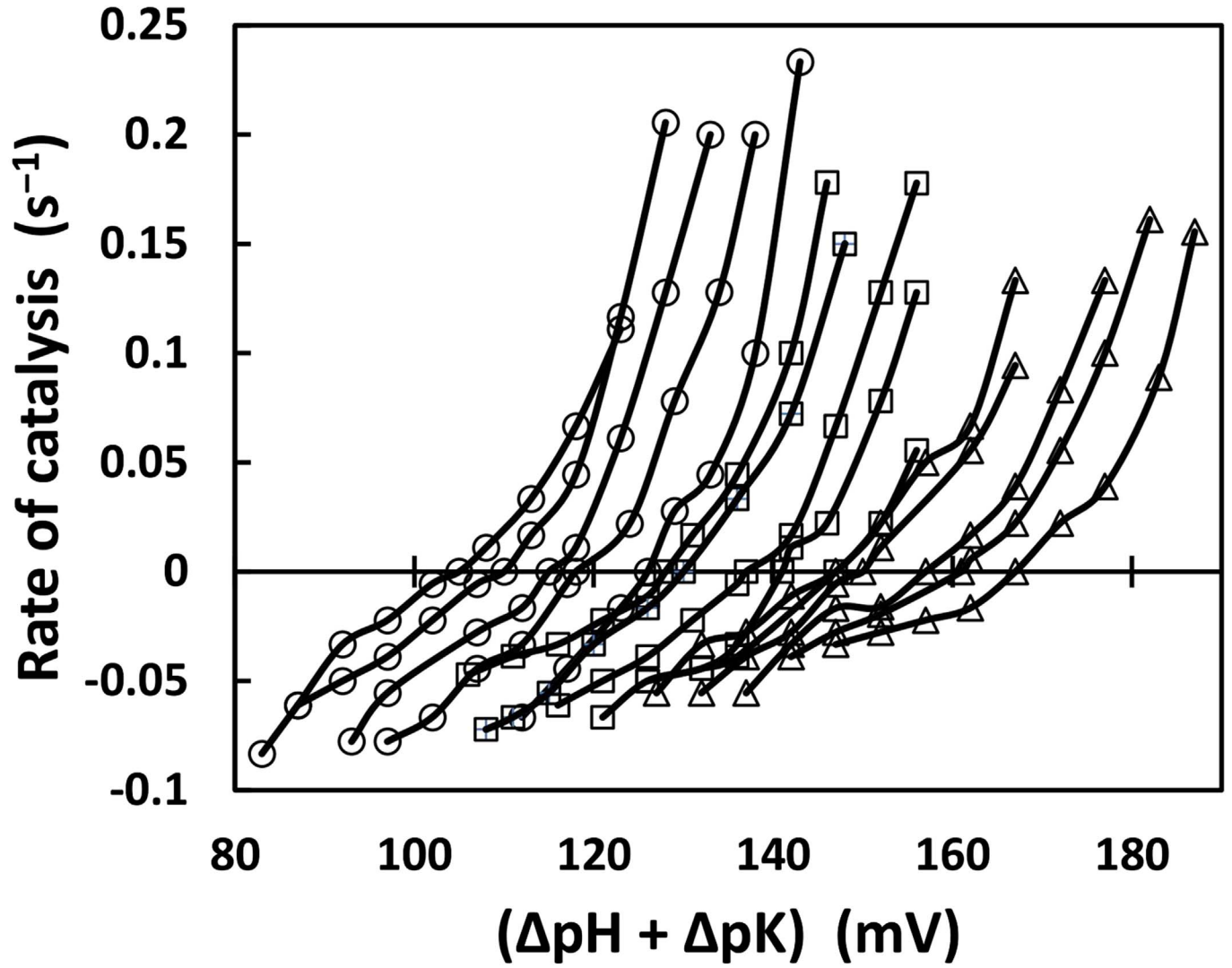


Fig. 2. The dependence of ATP synthesis/hydrolysis on (ΔpH + ΔpK) based on Eq. (17) describing the two-ion theory of energy coupling in Nath's torsional mechanism of energy transduction and ATP synthesis under various experimental conditions. [ATP] = 512 nM. [Pi] = 10 mM (○), 1 mM (□), or 0.1 mM (Δ). From left to right, [ADP] = 640, 320, 160, 80, and 40 μM for [Pi] = 10 mM (○), [ADP] = 320, 160, 80, 40, and 20 μM for [Pi] = 1 mM (□), and [ADP] = 320, 160, 80, 40, and 20 μM for [Pi] = 0.1 mM (Δ).

mediated potassium gradient was calculated from the measured $[K^+]_{in}$ and $[K^+]_{out}$ concentrations as being equal to $(pK_{in} - pK_{out}) = \Delta pK$. The results at different values of ΔpH and ΔpK and various combinations of ATP, ADP and Pi concentrations are tabulated in Table 1. For all combinations, the pH_{out} varied between 8.02 and 8.04, while the inside potassium concentration, $[K^+]_{in}$ was 5 mM [31].

According to the torsional mechanism, the true driving force for ATP synthesis is the sum of the electrochemical potential difference of proton and anion/countercation translocation. For the above situation, where the second ion in the process of coupling is the countercation K^+, the net driving force of ATP synthesis can be simplified as being equal to 2.303nRT(ΔpH + ΔpK) in energy units (e.g., kJ/mol) as shown by Eq. (17), where n is the number of translocations of the two ions per ATP, and n typically varies between 3 and 4, and equals 3.3 in the experiments of Soga et al. [31].

The calculations for all 28 cases are summarized in Table 1. The rate data under various conditions are nicely described by the above equation (Eq. (17)). The driving force in terms of (ΔpH + ΔpK) required to achieve net ATP synthesis (over hydrolysis) at various ADP, Pi and ATP concentrations is also tabulated in the last column of the table. The higher the [ATP]/([ADP][Pi]) ratio, the higher is the value of the last column. As a thumb-rule, for this energy-transducing TF_1F_O system, a driving force increase per translocation of H^+ and K^+ of approximately 0.7 kJ/mol is needed to push the system into synthesis mode for a doubling of the [ATP]/([ADP][Pi]) ratio. Further, it is found that at constant value of [ATP]/([ADP][Pi]), any combination of ΔpH or ΔpK yields superimposable rate curves, as long as the relationship (ΔpH + ΔpK) = constant is ensured. The numerical value of the minimum and maximum (i.e., range) of the K^+ gradient provided in the paper [31] is also tabulated in Table 1. The Table 1 is supplemented by a graph (Fig. 2) in which the measured rate of catalysis at various experimental conditions of ADP, Pi and ATP is plotted as a function of the overall driving force of ATP synthesis (ΔpH + ΔpK) (in mV), as interpreted by Eq. (17) of the two-ion theory.

Each of the fifteen curves in Fig. 2 reveals two regions or processes, separated by a point of inflection. The region of each curve where the net rate of catalysis, J is less than zero can be interpreted as being governed primarily by the process of ATP hydrolysis. In the second region (J > 0), net ATP synthesis driven by *both* H^+ and K^+ ion concentration gradients dominates, and in this region the rate of ATP synthesis varies parabolically as a function of (ΔpH + ΔpK) quite perfectly (Fig. 2). In fact, a fit of each of the fifteen curves by a second order polynomial in the region where predominantly net ATP synthesis occurs (i.e., J > 0) yielded a mean R^2 value of 0.995. An important

implication of this result is that an enzyme conformation with *two* different ionic species bound in the F_O portion of ATP synthase (H^+ and K^+ here, or H^+ and A^- in ATP synthases from other sources) is the only energetically competent and kinetically obligatory intermediate in ATP catalysis. In other words, the F_1F_O-ATP synthase is a *cotransporter*. Formulation of an enzyme kinetic model that mathematically describes the coupling of proton and co-anion/counter-cation transport with ATP synthesis would prove invaluable for future analysis of rate data in the field.

The meaning of the above results (Table 1, Fig. 2) is thus quite different from that inferred by conventional single-ion models of energy coupling such as the chemiosmotic theory, because here, two *different* ion species are contributing to the energy requirement of ATP synthesis. The clear-cut implication of this work is that the energy contribution to ATP synthesis by translocation of a second ion-type (e.g., K^+) cannot be slipped into the energy account of the H^+, as done by Soga et al. [31]; in fact the data is adequately explained by a governing equation of Nath's torsional mechanism of energy transduction and ATP synthesis [Eq. (8) or Eq. (17)]. The ion-coupling mode of translocation in Nath's torsional mechanism of ATP synthesis satisfactorily explains the details of energy coupling and transduction in ATP synthase.

It should finally be emphasized that the classical chemiosmotic theory is based on equilibrium thermodynamics (which is another of its major inadequacies and limitations), and in line with chemiosmosis, Soga et al. [31] have also interpreted their data based on the energetic equilibrium of the coupled reactions (i.e. generalized thermodynamic driving force, $X = \Delta G' = 0$). On the other hand, the torsional mechanism is a higher-level nonequilibrium theory [2,37] that employs nonequilibrium thermodynamics and kinetics in its description, and characterizes the nonequilibrium steady state of the coupled reactions occurring with a finite, non-zero net reaction rate of synthesis/hydrolysis. The last column in Table 1 (see also Fig. 2) describes a special case of a nonequilibrium *stationary* state (i.e. generalized flux, $J = 0$ in which the rate of ATP synthesis by a sub-population of the F_1F_O-ATP synthase enzymes in the proteoliposome system exactly balances the rate of ATP hydrolysis by (a different sub-population of) F_1-ATPase enzymes in the system carrying out ATP hydrolysis); however, in this analysis, the generalized thermodynamic force, X itself does not vanish as assumed by Soga et al. [31]. It should be emphasized that in this more general thermodynamic framework, equilibrium is a special *non-physiological* case of a nonequilibrium stationary state ($J = 0$) in which the generalized thermodynamic driving force also disappears, i.e. $X = 0$. The greater generality, applicability, and superiority for coupled biological processes of such a nonequilibrium analysis over the standard equilibrium treatment has been stressed repeatedly by several research groups over the decades ([3,4,10,12,17].

5. Conclusions

A fundamental flaw in the governing equations of Mitchell's chemiosmotic theory has been identified. Surprisingly, this flaw had not been detected earlier, despite the passage of more than half-a-century. Discovery of such a defect negates, or at least considerably weakens, the theoretical foundations on which the chemiosmotic theory is based. It is concluded that a two-ion theory of energy coupling within Nath's torsional mechanism of energy transduction and ATP synthesis rectifies the fundamental flaw in the chemiosmotic theory. The new paradigm has been shown to remove the inconsistencies in previous theories, and to provide the elusive mechanistic details of biological energy coupling, transduction, and ATP synthesis, a process on which experimental data is still extremely scarce (compared to the reverse process of ATP hydrolysis). It also possesses the power to rationalize and interpret experimental data and guide future experimental research and theoretical analysis in the field. The unifying principles of energy coupling and transduction contained in the torsional mechanism are of a very general and universal nature, properties that make these topics very attractive for contemporary interdisciplinary research. The aspects dealt with in the two-decade-long development and embellishment of the torsional mechanism can be taken to constitute the key elements whose lack of detailed consideration had held back the progress of research in this important field.

Acknowledgement

The funding and constant support extended by the Department of Science and Technology, India to the author's research program on the mechanism and thermodynamics of biological molecular machines for the past 25 years (1992–2016) is gratefully acknowledged. The author thanks all four reviewers for several comments and suggestions that have contributed to a logical explanation of the various mechanistic aspects addressed in the paper.

References

[1] B. Agarwal, Revisiting the 'chemiosmotic theory': Coupled transport of anion and proton for ATP synthesis, Bioenergetics 2 (2013) e116.
[2] C. Channakeshava, New paradigm for ATP synthesis and consumption, J. Biosci. 36 (2011) 3–4.
[3] R.S. Criddle, L.D. Hansen, B.N. Smith, et al., Thermodynamic law for adaptation of plants to environmental temperatures, Pure Appl. Chem. 77 (2005) 1425–1444.
[4] Y. Demirel, S.I. Sandler, Thermodynamics and bioenergetics, Biophys. Chem. 97 (2002) 87–111.
[5] T.G. Frey, C.A. Mannella, The internal structure of mitochondria, Trends Biochem. Sci. 25 (2000) 319–324.
[6] M. Ghochani, J.D. Nulton, P. Salamon, T.G. Frey, A. Rabinovitch, A.R.C. Baljon, Tensile forces and shape entropy explain observed crista structure in mitochondria, Biophys. J. 99 (2010) 3244–3254.
[7] D.E. Green, A critique of the chemosmotic model of energy coupling, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 2240–2243.
[8] L.D. Hansen, S. Jain, R. Murugavel, ATP synthase and the torsional mechanism: Resolving a 50-year-old mystery, Curr. Sci. 87 (2004) 16–19.
[9] T. Hornung, R. Ishmukhametov, D. Spetzler, J. Martin, W.D. Frasch, Determination of torque generation from the power stroke of *Escherichia coli* F_1-ATPase, Biochim. Biophys. Acta 1777 (2008) 579–582.
[10] D. Jou, F. Ferrer, A simple nonequilibrium thermodynamic description of some inhibitors of oxidative phosphorylation, J. Theor. Biol. 117 (1985) 471–488.
[11] E.A. Kasumov, R.E. Kasumov, I.V. Kasumova, A mechano-chemiosmotic model for the coupling of electron and proton transfer to ATP synthesis in energy-transforming membranes: A personal perspective, Photosynth. Res. 123 (2015) 1–22.
[12] G. Lebon, D. Jou, J. Casas-Vázquez, Understanding Non-equilibrium Thermodynamics: Foundations, Applications, *Frontiers*, Springer, Berlin, 2008.
[13] P. Mitchell, Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism, Nature 191 (1961) 144–148.
[14] P. Mitchell, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, Biol. Rev. 41 (1966) 445–502.
[15] P. Mitchell, Chemiosmotic coupling and energy transduction. In theoretical and experimental biophysics: A series of advances, volume 2 (Cole, A., Ed.), Marcel Dekker, N. Y. (1969) 159–216.
[16] P. Mitchell, J. Moyle, Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria, Nature 208 (1965) 147–151.
[17] S. Nath, A thermodynamic principle for the coupled bioenergetic processes of ATP synthesis, Pure Appl. Chem. 70 (1998) 639–644.
[18] S. Nath, H. Rohatgi, A. Saha, The torsional mechanism of energy transfer in ATP synthase, Curr. Sci. 77 (1999) 167–169.
[19] S. Nath, The molecular mechanism of ATP synthesis by F_1F_0-ATP synthase: A scrutiny of the major possibilities, Adv. Biochem. Eng. Biotechnol. 74 (2002) 65–98.
[20] S. Nath, Beyond the chemiosmotic theory: Analysis of key fundamental aspects of energy coupling in oxidative phosphorylation in the light of a torsional mechanism of energy transduction and ATP synthesis – Invited review part 1, J. Bioenerg. Biomembr. 42 (2010) 293–300.
[21] S. Nath, Beyond the chemiosmotic theory: Analysis of key fundamental aspects of energy coupling in oxidative phosphorylation in the light of a torsional mechanism of energy transduction and ATP synthesis – Invited review part 2, J. Bioenerg. Biomembr. 42 (2010) 301–309.
[22] S. Nath, Personal communication to E. C. Slater, C. Slater, June 2 and (June 4) (2010).
[23] S. Nath, J. Villadsen, Oxidative phosphorylation revisited, Biotechnol. Bioeng. 112 (2015) 429–437.
[24] S. Nath, Analysis of molecular mechanisms of ATP synthesis from the standpoint of the principle of electrical neutrality, Biophys. Chem. 224 (2017) 49–58.
[25] G.A. Perkins, M.H. Ellisman, Mitochondrial configurations in peripheral nerve suggest differential ATP production, J. Struct. Biol. 173 (2011) 117–127.
[26] N.M. Rao, Medical Biochemistry, 2nd ed, New Age International, New Delhi, 2014, pp. 280–281.
[27] B. Reynafarje, A. Alexandre, P. Davies, A.L. Lehninger, Proton translocation

stoichiometry of cytochrome oxidase: Use of a fast-responding oxygen electrode, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 7218–7222.
[28] E.C. Slater, Mechanism of phosphorylation in the respiratory chain, Nature 172 (1953) 975–978.
[29] E.C. Slater, The mechanism of the conservation of energy of biological oxidations, Eur. J. Biochem. 166 (1987) 489–504.
[30] E.C. Slater, Personal communication to S. Nath, Nath (June 4) (2010).
[31] N. Soga, K. Kimura, K. Kinosita, M. Yoshida, T. Suzuki, Perfect chemomechanical coupling of F_oF_1-ATP synthase, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 4960–4965.
[32] H. Tedeschi, Cell Physiology: Molecular Dynamics, 2nd edn, William C. Brown, Oxford, U.K., 1993.
[33] J. Villadsen, J. Nielsen, G. Lidén, Bioreaction Engineering Principles, 3rd edn, Springer, New York, 2011, pp. 137–145.
[34] G. Weber, Energetics of ligand binding to proteins, Adv. Protein Chem. 29 (1975) 1–83.
[35] R.J.P. Williams, Some unrealistic assumptions in the theory of chemi-osmosis and their consequences, FEBS Lett. 102 (1979) 126–132.
[36] R.J.P. Williams, Chemical advances in evolution by and changes in use of space during time, J. Theor. Biol. 268 (2011) 146–159.
[37] V. Wray, Commentary on Nath and Villadsen review entitled “oxidative phosphorylation revisited” Biotechnol. Bioeng. **112** (2015) 429-437, Biotechnol. Bioeng. 112 (2015) 1984–1985.
[38] L. Xu, F. Liu, The chemo-mechanical coupled model for F_1F_0-motor, Progress Biophys. Mol. Biol. 108 (2012) 139–148.
[39] R. Yasuda, H. Noji, K. Kinosita, M. Yoshida, F_1-ATPase is a highly efficient molecular motor that rotates with discrete 120° steps, Cell 93 (1998) 1117–1124.