Biophysical Chemistry 252 (2019) 106208

Contents lists available at ScienceDirect

Biophysical Chemistry

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

Integration of demand and supply sides in the ATP energy economics of cells

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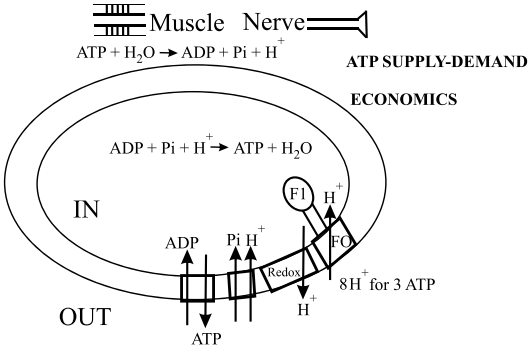
HIGHLIGHTS

- The mechanistic and operative P/O ratios in oxidative phosphorylation are determined.
- Both supply and supply-cum-demand sides of the ATP cellular economy are considered.
- Obtained values of P/O ratios are compared with experimental data.
- A molecular model of the process is presented.
- Chemical factors regulating coupling of cell respiration to ATP synthesis are proposed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Adenosine triphosphate (ATP)
Energy demand management/economics in biology
Oxidative phosphorylation
P/O ratio
Nath's torsional mechanism of energy transduction and ATP synthesis
Nath's two-ion theory of biological energy coupling
Coupling of ATP synthesis to oxygen consumption/respiration
Succinate
Dicarboxylic acids
Homeostasis
Metabolic regulation and control
Metabolic engineering

ABSTRACT

The central aspects of the energy economics of living cells revolve around the synthesis and utilization of molecules of adenosine triphosphate (ATP). Current descriptions of cell metabolism and its regulation in most textbooks of biochemistry assume that enzymes and transporters behave in the same way in isolation and in a cell. Calculations of the mechanistic or maximal P/O ratios in oxidative phosphorylation by mammalian cells generally consider only the supply side of the problem without linking to ATP-demand processes. The purpose of this article is to calculate the mechanistic P/O ratio by *integration of the supply and demand sides* of ATP reactions. The mechanistic stoichiometry calculated from an integrated approach is compared with that obtained from the standard model that considers only ATP supply. After accounting for leaks, slips, and other losses, the *actual* or *operative* P/O calculated by the integrated method is found to be in good agreement with the experimental values of the P/O ratio determined in mitochondria for both succinate and NADH-linked respiratory substrates. The thermodynamic consequences of these results and the biological implications are discussed. An integrated model of oxidative phosphorylation that goes beyond the chemiosmotic theory is presented, and a solution to the longstanding fundamental problem of respiratory control is found.

1. Introduction

An important goal of metabolic modeling is to link bioenergetics with the thermodynamics of chemical reactions occurring in cells [1–6]. To the best of the author's knowledge, the fundamental question of how exactly cell respiration is regulated in response to alterations in energy demand in the cell has not been unequivocally answered, and the factor(s) determining such regulation at the molecular level have not been unambiguously characterized. Adenosine triphosphate (ATP) can be seen as the energetic factor that links anabolism and catabolism,

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https://doi.org/10.1016/j.bpc.2019.106208
Received 29 April 2019; Received in revised form 14 June 2019
Available online 17 June 2019

and also drives the important processes of muscle contraction, synaptic nerve transmission, and active transport [6–8]. Hence ATP connects energy-producing and energy-consuming processes and is therefore key to solving the enigma of matching energy supply and demand in the cell and to tune the control and regulation mechanisms for optimal function [9–11]. The P/O ratio is a key factor in determining the coupling between the redox and ATP sides in the fundamental process of oxidative phosphorylation and thereby cellular function [1,2,5,6,12–14].

The chemiosmotic theory was opposed to conformational mechanisms [15–17] and considered both the H^+/O ratio and the H^+/ATP ratio to be small integers, with the mechanistic P/O as the ratio of the above stoichiometries. However, alternative mechanisms [18–20] offer important information on the stoichiometry of coupling (see Sections 2, 3).

The discovery of inter-subunit rotation in F_1-ATPase by direct visualization using epifluorescence microscopy techniques [21], and the presence of symmetry mismatch between F_O and F_1 in ATP synthase [22] necessitated the formulation of a new molecular mechanism of energy coupling in ATP synthase [23–26]. Over the past two decades, the mechanism, named Nath's torsional mechanism of energy transduction and ATP synthesis by other authors, has been further embellished and woven into a consistent theory that integrates all the available information in the field [9–11, 18–20, 23–29]. Various aspects of the theory can also be understood by gleaning from the commentary articles, book extracts, and other material written around it by other scientists [6,13,14,30–38].

Key kinetic aspects of the process of ATP synthesis were addressed previously at the time of inception of the torsional mechanism [24]. It ought to be stressed that the fundamental thermodynamic aspects of the process represent vital attributes of no lesser significance. In particular, the calculation of the mechanistic and actual P/O ratio in mammalian cells integrating the supply and demand sides and all the available experimental information is central to the cellular economics involving ATP, and is a principal aim of this work.

2. Results

The maximum ATP yield from oxidative phosphorylation per oxygen atom consumed, the mechanistic P/O ratio using isolated mitochondria as the experimental material, has been the subject of a large number of investigations for over 80 years. For the first ~50 years, the mechanistic P/O ratio was thought to be an integer, with a maximum value of 3 for oxidation of NADH-linked substrates, and 2 with succinate as substrate. With the advent of the chemiosmotic theory [15,16], the mechanistic P/O was seen as the ratio of the number of protons pumped out of the matrix by the respiratory chain (H^+/O) and the number of protons required to synthesize one ATP molecule (H^+/ATP). In the chemiosmotic framework, both the H^+/O ratio and the H^+/ATP ratio were considered to be integers, with each integer value arising from the underlying molecular mechanisms on the redox and ATP sides respectively. However, the ratio of H^+/O and H^+/ATP that yielded the mechanistic P/O is not necessarily an integer value.

Following several pioneering experimental advances in the structure of the membrane-bound F_O portion of ATP synthase by X-ray and cryo-EM techniques in the new millennium, it was found that the H^+/ATP ratio need not be an integer. In other words, the number of c-subunits in the c-oligomer of F_O was no longer constrained to be a multiple of 3, and a new theory, such as Nath's torsional mechanism of energy transduction and ATP synthesis [9,10,19,23–29] was required to rationalize the new information. The mechanism was shown to work whether there was symmetry or there existed a symmetry mismatch between F_1 and F_O in ATP synthase [pp. 1792–1794 in ref. 28].

2.1. Supply-side calculation of the mechanistic P/O ratio

In mammalian mitochondria, the consensus value of the number of c-subunits in the c-ring of F_O is eight. The consensus value of the oxidative phosphorylation H^+/O ratio for NADH-linked substrates is ten, and six for succinate. The standard supply-side calculation of the mechanistic P/O ratio in mitochondria proceeds as follows (Fig. 1). A value of $8H^+$ is taken to be translocated through the F_1F_O-ATP synthase per 360° rotation, which leads to the synthesis of 3 ATP molecules. An additional proton is taken to be translocated through the adenine nucleotide translocase and phosphate carrier from the cytosol to the matrix ("out to "in" in Fig. 1) per ATP made. Thus, in the calculation, $8H^+$ are translocated through the ATP synthase and $3H^+$ are translocated through the adenine nucleotide and phosphate carriers for every 3 ATP molecules synthesized, yielding an H^+/ATP ratio of 11/3. Hence the mechanistic (maximum) P/O ratio for NADH-linked substrates based on supply-side economics works out to be $H^+/O \times ATP/H^+ = 10 \times 3/11 = 2.727$, while the $(P/O)_{max}$ for oxidation of succinate by mitochondria is predicted to be $6 \times 3/11 = 1.636$.

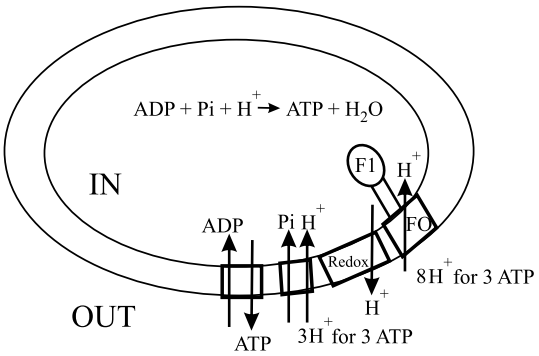

Fig. 1. Schematic diagram for calculation of P/O ratios for oxidative phosphorylation by mammalian cells based on supply side ATP economics.

2.2. Integration of elementary transport and catalysis steps in ATP synthesis mechanism and integrated demand-side and supply-side calculation of the mechanistic P/O ratio

Looking at only the supply-side equation in the calculation of the mechanistic P/O ratio (Section 2.1) by the standard model is inherently flawed. This arises from the failure of the standard model to consider the demand side of the equation [3,4,24]. From the inception of the torsional mechanism, it had been postulated that in vivo in the cell, the demand and supply sides are inextricably linked and that "the supply of ATP by the process of ATP synthesis and transport is regulated by the demand for ATP for various cellular processes occurring elsewhere in the cell" [9,24,26,28]. In other words, the metabolic flux or rate in a cellular pathway increases because the ATP demand and ATPase rate in various ATP-utilizing processes in the cell increases, for example by nerve synaptic activity or muscular contraction (Fig. 2). Conversely, it is also true that if the overall metabolic/ATP synthesis rate increases, it implies that the steady state sum of ATPases has increased. Hence it is imperative to consider both demand and supply sides in an integrated way for the regulation and management of cellular energy economics.

As discussed above, ATP synthesis was considered to be regulated by the demand for various cellular processes [9,24,26,28]. The specific aspects of regulation of the steps of transport and catalysis in the oxidative phosphorylation process were visualized in the following way. When ATP^{4-} is required, it is transported out from the mitochondrial matrix/intracristal space to the cytosol ("in" to "out" in Fig. 2) along its concentration gradient and exchanged for ADP^{3-} that moves from "out" to "in" along its concentration gradient through the adenine nucleotide translocase. The resulting unbalanced *local* electrical potential is the signal that causes HPO_4^{2-} to translocate from "out" to "in" (Fig. 2) along its concentration gradient and is exchanged for an OH^- produced during ATP synthesis in the F_1 portion of ATP synthase from "in" to "out", or what is electrically equivalent, in symsequenceport

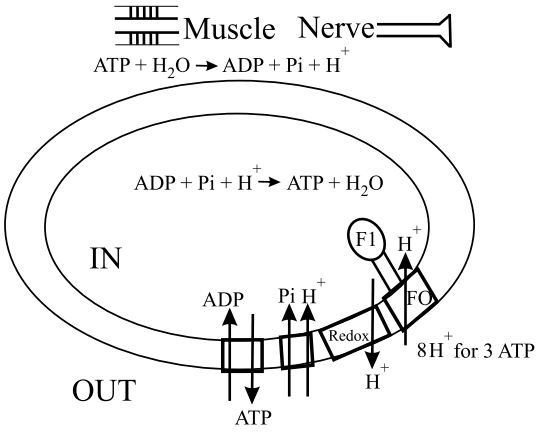

Fig. 2. Schematic diagram for calculation of P/O ratios for oxidative phosphorylation by mammalian cells based on steady-state integrated supply and demand side ATP economics. In metabolically intact nerve synapses, the energy demand for synaptic function results in ~10^6 ATP molecules per nerve terminal at steady state [8]. Breast muscles of the hummingbird during flight have a steady state energy demand rate of 500–600 μmol ATP $g^{-1}\,min^{-1}$, which is > 500 times their resting metabolic rate [78].

with an H^+ ion from "in" to "out" by the phosphate carrier as depicted in the simplified representation of Fig. 2a and b. We require 8 protons to synthesize 3 ATP molecules by the mammalian F_1F_O-ATP synthase by the ATP synthesis reaction (Eq. (1)).

$$ADP^{3-} + HPO_4^{2-} + H^+ = ATP^{4-} + H_2O \quad (1)$$

However, in the in vivo demand process in the cytosol that consumes the ATP transported out of the mitochondrion, the reverse reaction operates, i.e. the ATP consumes an OH^- and is cleaved into ADP and Pi, or, as shown by Eq. (2), the ATP hydrolysis reaction produces an H^+ ion per ATP molecule cleaved.

$$ATP^{4-} + H_2O = ADP^{3-} + HPO_4^{2-} + H^+ \quad (2)$$

Thus we need only 8 protons (and not eleven) to synthesize 3 ATP molecules by the mammalian F_1F_O-ATP synthase in the chemical reaction of ATP synthesis, i.e. H^+/ATP = 2.67. In each cycle of oxidative phosphorylation, per oxygen atom or per pair of electrons, 10 protons are pumped out by the redox complexes with NADH-linked substrates (H^+/O = 10) and 6 protons are translocated from the matrix to the intracristal space against the concentration gradient with succinate as substrate (H^+/O = 6).

The calculation of the mechanistic P/O ratio in mammalian cells by the integrated approach works as follows (Fig. 2). $8H^+$ are translocated (from "out" to "in") through the access half-channels in the F_O portion of the ATP synthase leading to the synthesis of 3 ATP molecules by the reaction of Eq. (1). However, as explained above, the three additional transport protons per 3 molecules of ATP are now provided by the reverse ATPase reaction (Eq. (2), Fig. 2) on the *demand side* that fuel the demand for ATP synthesis in the first place, and do not need to be added in the energy balance as in Section 2.1. This is because the three additional transport protons are not translocated through the access half-channels in the membrane-bound F_O-portion of the ATP synthase and therefore do not donate energy to synthesize the ATP. Hence the mechanistic (maximum) P/O ratio for NADH-linked substrates in the case where the demand and supply sides are linked works out to be H^+/O × ATP/H^+ = 10 × 3/8 = 3.75, while the $(P/O)_{max}$ for oxidation of succinate by mitochondria is predicted to be 6 × 3/8 = 2.25.

2.3. Actual P/O ratio and actual yield of ATP from glucose

The actual, operating P/O ratio in the cell will be quite different from the mechanistic or maximum P/O ratio calculated in Sections 2.1 and 2.2. The presence of leak in the membrane, of slip in the pumps, active transport and other losses cause energy to be lost, depending on the tissue and the physiological conditions, thus lowering the actual or operating P/O in the cell from $(P/O)_{max}$. Thus, unlike the value of the mechanistic P/O ratio, it is inevitable that the values of the operating P/O ratio under real physiological conditions are *variable* within limits, depending on the magnitude of the losses. These losses have been carefully estimated earlier from four different approaches, including by use of the detailed thermodynamic model based on the torsional mechanism, and found to be consonant [9].

Thus, while the mechanistic, ideal, zero loss P/O ratio can take on two *constant* values by theory ($(P/O)_{max}$ of 3.75 for NADH-linked substrates, and 2.25 for succinate as substrate), as calculated in Section 2.2, the actual operating P/O ratio under physiological conditions, $(P/O)_{actual}$, will be *variable*, with its value based on several conditions and the extent of the various losses. These losses have been estimated earlier [9]. Employing consensus values for losses such as leaks and slips, the estimates of the actual P/O in the cell considering linked supply and demand sides as proposed by the torsional mechanism and solely the supply side as in the standard model are tabulated and compared with experiment in Tables 1 and 2 for both succinate and NADH-based oxidation substrates. Based on Nath's torsional mechanism, the actual yield of ATP for complete oxidation of glucose works out to be 32 ATP/glucose, assuming the *consensus* value of the actual, operating P/O ratio of 2.5 under physiological conditions for NADH-based substrates. It should be emphasized that if higher values of the operating P/O are determined under real, physiological conditions, values that have been obtained at times by experimental investigators (Table 1), then, correspondingly, the actual yield of ATP will go up from 32 ATP/glucose, i.e. it can be variable too. Similarly, for leaky mitochondria or in mitochondria where the respiratory control ratio (RCR) is low, i.e. in general for not so well-coupled systems, the operative P/O ratio can be lower than the consensus values, and the ATP per glucose will be lower than 32. In fact, Fig. 1 of ref. 9 shows that depending on the pH, a wide range for losses is possible, if inefficient operation with respect to ATP production is permitted. An example of biological significance in this

Table 1
Predicted maximum and actual P/O ratio for oxidative phosphorylation in mammalian cells on NADH-linked substrates and comparison with experimentally determined values.

Standard model			Nath's torsional mechanism [9]			Experimental $(P/O)_{actual}$
$(P/O)_{max}$	$(P/O)_{actual}$ with 20% leak and 10% slip	$(P/O)_{actual}$ including all losses (η = 67%)	$(P/O)_{max}$	$(P/O)_{actual}$ with 20% leak and 10% slip	$(P/O)_{actual}$ including all losses (η = 67%)	2.3–3.0 [45] 2.51–2.73 [61] 2.7–2.9 [62] 2.6 [63] 2.3 [64] 2.5 [65] 2.5 [66] 2.9 [67] 2.25 ± 0.05 [73] 2.44 ± 0.12 [74]
2.727	1.91	1.83	3.75	2.625	2.51	

Table 2
Predicted maximum and actual P/O ratio for oxidative phosphorylation in mammalian cells with succinate as substrate and comparison with experimentally determined values.

Standard model			Nath's torsional mechanism [9]			Experimental $(P/O)_{actual}$
$(P/O)_{max}$	$(P/O)_{actual}$ with 20% leak and 10% slip	$(P/O)_{actual}$ including all losses (η = 67%)	$(P/O)_{max}$	$(P/O)_{actual}$ with 20% leak and 10% slip	$(P/O)_{actual}$ including all losses (η = 67%)	1.48–1.62 [10] 1.5 [45] 1.62–1.78 [61] 1.6–1.8 [62]
1.636	1.14	1.10	2.25	1.575	1.51	1.5 [63] 1.5 [64] 1.8 [67] 1.4–1.8 [68] 1.3–1.5 [69] 1.3–1.5 [70] 1.7 [71] 1.6 [72] 1.50 ± 0.14 [75]

regard is illustrated by mitochondria from brown adipose tissue that permit nonshivering thermogenesis, essential for the survival of many mammals. Such systems would be characterized by low operating P/O values, low thermodynamic efficiency of coupling, and high rates of dissipation of free energy, although a quantitative treatment appears to be lacking. In summary, there is no difficulty for the integrated model to accommodate various operating stoichiometries. The outliers in Tables 1, 2 can be accounted for based on the experimental design, the experimental conditions used, the range of process variables employed, or by the lack of corrections for systematic errors occurring in the measured values.

Typically, tightly-coupled mitochondria exhibit values of RCR from five to ten. Thus, in these systems the fraction of leak can vary between 10% and 20%. For neuronal mitochondria, a value of 20% has been employed as the standard loss value arising from leak, and P/O ratios have been "downgraded" by this loss percentage as a matter of operational convention (Prof. William Levy, University of Virginia, personal communication, May 2019). Similarly, for well-coupled systems optimized for ATP synthesis, the fraction of slip can also vary approximately between 10% and 20% [9]. The selected leak and slip fractions in Tables 1 and 2 are for illustrative purposes for the case of well-coupled systems. However, the difference between the standard and integrated models will remain for other values of losses within this range for well-coupled systems. Thus the results summarized in Tables 1 and 2 are not qualitatively altered by selection of other likely values of percent losses in these systems. In addition, for a more complete analysis, other losses, e.g. those arising due to active transport of ions are also included in the calculation that takes η = 67%.

The actual or operating P/O has been calculated *theoretically* by three different approaches, a) by a fundamental nonequilibrium thermodynamic analysis, b) novel insights arising from Nath's torsional mechanism of energy transduction and ATP synthesis, and c) by the overall balance of cellular energetics, and was shown to lead to consonant results [9]. Hence it is justified to compare these estimates obtained by theory with the experimental determinations of the operating P/O for mitochondrial systems as done in Tables 1 and 2. The consensus values of the operating P/O of 2.5 (1.5 with succinate) are in agreement with other estimates [e.g. 64], and also corroborated by a founder of the field (Prof. E.C. Slater, Lymington, U.K. and University of Amsterdam, personal communication, 2007). The central issue appears to be with the approach used to calculate the mechanistic P/O ratio (standard model vs. integrated model) and the accounting method used for quantifying protons. The accounting method of the standard supply-side approach (Section 2.1) uses 11 protons per 3 ATP, while that by the integrated demand cum supply method employs 8 protons to synthesize 3 ATP molecules and maintains a true steady state. The H^+-accounting method of the standard model had not been checked for its correctness and the calculation of the theoretical/mechanistic values themselves had not been scrutinized until this work but had been used blindly for decades, without a true understanding of their origins. The error can thus be attributed to "a historical burden," an interesting topic for further exploration by historians and philosophers of science.

The values of the mechanistic (theoretically maximum) P/O are also summarized in Tables 1, 2. The results show conclusively that mechanistic P/O ratios are not very useful tools for analyzing ATP synthesis under real physiological conditions, and use of actual P/O ratios offer a superior alternative (Tables 1, 2). The consequence of these and other results and the biological implications arising are discussed in Section 3.

A key factor determining the actual or operative P/O ratio is the prevailing pH in the intracristal space/matrix of mitochondria. A quantitative analysis of the dependence of the magnitude of the losses on the prevailing pH in the intracristal compartment has been carried out previously [see Fig. 1 in ref. 9]. It has generally been recognized that mitochondrial leaks and slips are important contributors to the total energy loss in mitochondrial energy transduction. However, it should be noted that recent results show that the pH of the intracristal space is a major determinant of the magnitude of the energy losses due to mitochondrial leaks and slips [9]. Some of the other factors that can contribute to the losses referred to above, and thereby to the *variability* of the actual or *observed* P/O ratio, are the type of substrate, organism and age, cell type, presence of side reactions, and temperature. Unfortunately, reported values of the operating P/O in the literature have often appeared as single values, with no mention of error, or as ranges (Tables 1, 2). Hence a statistical analysis for meaningful comparison could not be performed.

3. Discussion

3.1. Predicted mechanistic and actual P/O ratios by various models and their comparison with experiment

The mechanistic (ideal, i.e. maximum, zero loss) values of the P/O ratio based on various models have been calculated in Section 2 and summarized in Tables 1 and 2. The values of $(P/O)_{max}$ are 2.727 and 1.636 by the standard model for oxidation on NADH-based substrates and succinate respectively. The values of $(P/O)_{max}$ are 3.75 and 2.25 for NADH-linked substrates and succinate respectively according to the torsional mechanism. The values of the actual (operating) P/O ratio are also given in the Tables. As explained in Section 2.3, the values of $(P/O)_{actual}$ can be variable depending on the extent of the various losses and the method employed for their calculation or measurement. When all losses are included, they work out to be 1.83 and 1.10 with NADH-linked substrates and succinate respectively as per the standard model, and 2.51 and 1.51 with NADH-linked substrates and succinate respectively by the torsional mechanism [9] (Tables 1 and 2). The last column

in Tables 1 and 2 collects the experimentally determined operating P/O ratio obtained by several investigators over the decades. It is found that calculations based on the torsional mechanism agree reasonably well with the experimental determinations for oxidation on both NADH-linked substrates and succinate. On the other hand, predictions based on the standard model considerably underestimate the experimental values, with mean errors relative to experiment of over 28% for NADH-linked substrates and > 30% with succinate as substrate.

3.2. Experimental P/O ratios with isolated mitochondria

The calculations of actual P/O ratios as per the torsional mechanism also correspond to the experimental determinations, collated in Tables 1 and 2, of P/O ratios in state 3 on isolated mitochondria. The oxidative phosphorylation redox complexes and the ATP synthasome are localized in the cristae membrane, with the F_1-sector of ATP synthase protruding into the mitochondrial matrix space. [39–43]. The chemical reaction of ATP synthesis on F_1 (Eq. (1)) occurs in the matrix, and the OH^- produced during ATP synthesis in the F_1 portion of ATP synthase is readily exchanged with the inorganic phosphate by the phosphate carrier. Thus, the H^+ entering the intracristal compartment can readily react with the OH^- to form water leading to swelling of the cristae, for which there is structural evidence [40,44]. Alternatively, water can be formed in the cytosol, if the neutralization reaction occurs outside the mitochondria. The presence of this extra compartment or the formulation of more complex schemes than those illustrated in Figs. 1, 2 do not alter the results of Section 2. In fact, these "transport" or "neutralization" protons (3 per 3 ATP molecules) are not "*coupling*" protons, in that they are not linked to oxygen consumption or to translocation from matrix to intracristal space by the redox pumps. Nor are these protons translocated through the access channels in the F_O portion of ATP synthase; therefore they are not involved in energy coupling and do not contribute energy to actual synthesis of the ATP molecule.

The important point from the above discussion is that the P/O experiments on isolated mitochondria operating in state 3 conditions determine the ADP phosphorylated to ATP and the oxygen consumed during the phosphorylation by the oxygen electrode method pioneered by Chance and Williams [45,46], followed subsequently in many studies. These studies do not obtain the P/O by the calculations of the standard model outlined in Section 2.1, and they do not follow H^+ movements. Thus, they determine the P/O by *direct* measurements of ADP consumed (or ATP synthesized) and the total oxygen consumed, and therefore they have no need to determine the P/O as the ratio of (H^+/O) / (H^+/ATP). Since only the "coupling" protons that are translocated between matrix and intracristal space by the redox enzymes and the ATP synthase are linked to oxygen consumption and are competent to produce ATP, the experimental studies on isolated mitochondria by the oxygen electrode – ADP method measure the *actual* P/O ratio under state 3 physiological conditions (Tables 1, 2). The three extra protons per 3 ATP (named the "transport" or "neutralization" protons above) are not involved in the coupling of oxidation to ATP synthesis, and therefore *these protons are not involved in donating energy for the production of ATP*.

3.3. Incorporation of Mg^{2+} in the reactions

The catalytic role of Mg^{2+} in ATP synthesis is central and crucial [25,47], given that MgADP is the true substrate of ATP synthesis in F_1. However, for our purposes, use of $MgADP^-$ and $MgATP^{2-}$ in Eq. (1) and Eq. (2) does not alter any of the results of Section 2 or Tables 1, 2. ADP^{3-} and ATP^{4-} are chosen to be used in Eqs. (1), (2) because the adenine nucleotide translocator exchanges free ATP for free ADP, but not the Mg^{2+} bound forms.

3.4. Molecular explanation of leaks and slips

The torsional mechanism provides a *molecular* explanation of leaks and slips [9,10], which, along with the motive process, had been treated previously by macroscopic approaches [1,2,5,6,12,13,30,48–51]. In mitochondrial energy coupling, leaks, slips and the motive ionic species are all various forms of the dicarboxylic acid succinate, which is involved in a cotransport with protons. Thus, $[H_2A]$ is the neutral, undissociated form of succinic acid ("leak", taken as 20% here at the physiological conditions of temperature, and especially pH prevailing in the mitochondrial intracristal space [Fig. 1 of ref. 9]) that can freely diffuse through the energy-transducing crista membrane and is uncoupled from ATP synthesis by F_1F_O-ATP synthase. This circulation of $[H_2A]$ is also seen as the principal source of the resting state 4 respiration in mitochondria [10]. The di-ionic form, $[A_2^-]$ is pumped out along with protons by the redox complexes ("slip", taken as 10% here in Tables 1 and 2), but is impermeant to the F_O portion of ATP synthase. Only the motive, mono-ionic $[HA^-]$ form in symsequenceport with protons is competent to synthesize ATP by the F_1F_O-ATP synthase. A homeostasis of all three forms of the dicarboxlic acid is required to maintain a *nonequilibrium steady state* during physiological oxidative phosphorylation. A similar molecular model is proposed for photosynthesis, but with malic acid as the dicarboxylic acid selected by nature [10].

Based on values of the RCR from five to ten typically exhibited by well-coupled mitochondria, the fraction of leak can vary between 10% and 20%. Similarly, the fraction of slip can also vary between about 10% and 20% [9]. The selected leak and slip fractions in Tables 1 and 2 are for illustrative purposes for the case of well-coupled systems. However, the difference between the standard and integrated models will remain for other values of losses in this range. Thus the results summarized in Tables 1 and 2 are not qualitatively altered by selection of other values for percent losses in these systems. Moreover, analysis of the dicarboxylic acid – proton systems in which the mono-anion is the motive species and the neutral and di-anionic species constitute loss in the form of leak and slip respectively explains why *losses can never be completely eliminated but can only be minimized*. This arises from physical chemistry considerations which show that one cannot have 100% mono-anionic species in dicarboxylic acid systems under any process conditions [9].

3.5. Novel solution to the problem of respiratory control: succinate as the elusive signalling molecule involved in "multisite regulation" of oxidative phosphorylation and the tricarboxylic acid cycle

Given the results in a recent analysis of the state 3 to state 4 transition [10,11], the signalling for increased redox activity, oxygen consumption, and increased turnover of the Krebs cycle in response to enhanced rate of ATP synthesis in mitochondria implies the need for joint, parallel "multisite activation and regulation" of the pathway. How is the signal for enhanced upsteam activity communicated upon increased ATP production downstream? This signalling can only be achieved by a molecule that is translocated back and forth from mitochondrial matrix to cristae and senses and reports the enhanced rate of ATP synthesis by binding to complexes on the redox side and to a component of the Krebs cycle, yet is itself not consumed in the pathway or transported out of mitochondria.

Previous solutions to the problem have proposed ADP and Pi as feedback signals that are responsible for energy supply-demand matching in mammalian heart and in muscle [7,52–55]; however, these species are consumed in the reaction ((Eq. (1)), and hence are not available to act as signals of respiratory coupling. ATP itself has been proposed as a possibility [56]; however, ATP is immediately transported out of mitochondria, and is therefore not present to signal changes to the redox side of oxidative phosphorylation and the Krebs cycle. Considerable attention has been focused on K^+ [57] (Section

3.6), and especially on Ca^{2+} as the signalling molecule in nerve and muscle [7,8,55,58]. However, Ca^{2+} is not common to all the complexes and has not been reported to be translocated to and fro between matrix and cristae during physiological ATP synthesis. Protons do meet some of the above requirements, but they are not efficient signalling molecules in an alkaline pH 8 medium of the matrix, and therefore cannot be a suitable candidate.

Ideally, the candidate must be one of the regenerating substrates of the Krebs catalytic cycle, so that an increase in its concentration can increase the rate of the catalytic cycle. Further, the molecule should connect and be common to all the complexes involved in the pathway: the ATP synthasome, the redox complexes, and the Krebs cycle. It is proposed here for the first time that succinate is that elusive signalling molecule, *X*, and in fact, it is the only possibility that meets all the requirements listed above according to Nath's two-ion theory of energy coupling [10,20,26,59,60]. Thus only a "multisite activation" by succinate binding can ensure tight regulation in oxidative phosphorylation along with maintenance of a more or less constant steady state ratio of $NADH/NAD^+$ and approximately constant steady state concentrations of ADP, ATP, Pi and other metabolites.

3.6. Cation vs. anion translocation in mitochondria

The obvious question arises why considerably greater attention has been accorded in the literature to cation transport in mitochondria, compared with the rapid translocation of common anionic metabolites with which mitochondria, on teleological grounds, should be far more concerned? A possible reason could be that under in vitro conditions, as in the cycling of K^+ in valinomycin-treated mitochondria, it has been readily demonstrated that energy-driven cation gradients can be established [57]. Thousands of such studies with valinomycin as ionophore have been performed over the decades. However, the concentration of cytosolic K^+ (100–120 mM) is in the same range as that estimated inside isolated mitochondria. Hence it is very hard to conceive that the establishment of such cationic gradients is the true in vivo function of the transport mechanism. The principal translocative function of mitochondria should be the maintenance of a bidirectional transport flux of the various *anionic* metabolites instead of the relatively pointless translocation of cations. Thus, the addition of a foreign ionophore such as valinomycin may have unveiled the capacity of a pump/transporter for moving cations.

Untreated mitochondria are normally more permeable to anions than to cations. The addition of a foreign antibiotic such as valinomycin would be expected to convert/hijack a transport system normally involved in vivo in the rapid translocation of metabolite anions (such as succinate) into one that catalyzes the rapid transport of cations through a barrier with low cationic permeability. Such a mechanism would rationalize a large number of known mitochondrial properties.

3.7. Discussion of the nonequilibrium thermodynamic work of Stucki (1980)

As desired by one of the reviewers, aspects of the nonequilibrium thermodynamic analysis of oxidative phosphorylation by Stucki [76] are discussed in this Section. Stucki was primarily concerned with finding optimality criteria for linear energy converters, such as output flow, output efficiency, output power, economic output flow, and economic output power. It ought to be stressed that he did not determine the mechanistic stoichiometry of ATP synthesis in his work [76], but rather assumed the then prevalent integer value of 3 for NADH-based substrates. Based on this assumed stoichiometry of 3, he obtained an optimal operating P/O ratio of 2.36 for oxidative phosphorylation. Re-calculation of this value using the mechanistic P/O ratio of the standard model or that of the integrated model yields respectively an underestimated value of the optimal operative P/O ratio of 2.14, or an overestimated value of 2.95 compared to experiment (Table 1). The importance of Stucki's work has been covered previously [10–12]. However, the deficiencies in his thermodynamic analysis have not been addressed earlier.

(i) Stucki's results and entire analysis hinge on a "conductance matching" criterion (Eq. (36) in ref. 76) that needs to be satisfied at every degree of coupling, q. This equation stipulates that

$$\frac{L_{33}}{L_{PP}} = \sqrt{1 - q^2} \tag{3}$$

where L_{33} is the phenomenological conductance of an unspecified load(s) that is further assumed to be driven only by the phosphate potential [76], L_{PP} is the phenomenological conductance of ATP synthesis, and q is the degree of coupling of oxidative phosphorylation driven by respiration. However, the validity of Eq. (3), which is the necessary and sufficient condition for optimal efficiency in Stucki's work [76] has not been proved, and no rationale has been provided for it to hold at each and every degree of coupling. Hence the foundations of Stucki's analysis are considerably weakened.

(ii) The operating steady state of oxidative phosphorylation is proposed to satisfy Prigogine's principle of minimum entropy production [Eqs. (34), (36) of ref. 76], despite the fact that the basic conditions of Prigogine's analysis, namely the disappearance of the driven thermodynamic flow ($J_P = 0$) and the presence of an open-circuited driving force at static head are no longer satisfied.

(iii) In contrast to Stucki's finding of minimum dissipation (Φ) at the optimal steady state [76], a maximum Φ has been obtained for the operating steady state in oxidative phosphorylation, given the constraints [10,11,51,77].

(iv) In Stucki's analysis, the variation of efficiency, η and other output functions are plotted as a function of a *normalized* force ratio Zx with q as parameter. Here, Z is the phenomenological stoichiometry, not necessarily equal to the mechanistic stoichiometry used in this work, and x is the affinity force ratio of the phosphorylation and oxidation reactions. However, in experiments one measures the thermodynamic forces and the affinity force ratio, x, while Z is a calculated parameter. Only after Z is calculated can the η vs. Zx plot be made. If Z were simply a normalization constant, Stucki's procedure may perhaps be rationalized. However, Z itself is a function of the degree of coupling, q, a possibility that has been neglected in Stucki's analysis [76]. There is also no basis for normalization of the output functions (e.g. output flow, output power etc.) by these q-dependent factors. In fact, the dependence of Z on q is different depending on how uncoupling is brought about, e.g. by leak or by slip, i.e. it is dependent on the mechanism of uncoupling [60]. These difficulties are circumvented by Nath's nonequilibrium thermodynamic analysis of ATP synthesis [10,11] where the efficiency of energy conversion, η and the variation of output functions are studied as a function of the actual force ratio, x.

(v) Finally, all previous irreversible thermodynamic analyses of oxidative phosphorylation use a 2×2 conductance matrix, and do not take into account *direct* coupling between oxidation and phosphorylation because they fix $L_{OP} = 0$. These frameworks cannot explain the well-known phenomenon of respiratory control in oxidative phosphorylation (Section 3.5).

3.8. Integrated model for oxidative phosphorylation

As discussed in Section 3.5, the phenomenon of respiratory control is one of the key fundamental experimental observations that relates to the molecular mechanism of oxidative phosphorylation. The control exercised by the phosphorylation reaction on the rate of respiration strongly indicates the presence of *direct* coupling between the two reactions. The process of uncoupling of ion transport from ATP synthesis [59,60] when the control of the ATP reaction on respiration is

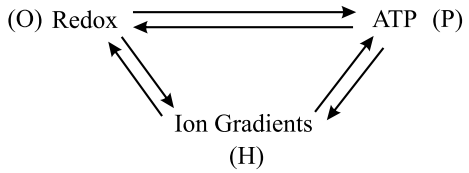

Fig. 3. Coupling scheme for oxidative phosphorylation based on the integrated model developed in this work.

weakened or completely released also shows the importance of coupling in control of respiration rates and the role of energized ionic intermediates in the coupling process. Any model of oxidative phosphorylation has to be able to explain both these phenomena in a compatible way.

An integrated model of oxidative phosphorylation based on the considerations in this work is given in the scheme of Fig. 3. Each connection in Fig. 3 indicates the existence of a coupling mechanism. Within the framework of irreversible thermodynamics, the processes of oxidation, ATP synthesis and ion transport are driven respectively by the oxidation affinity A_O, the phosphorylation affinity A_P, and the electrochemical potential difference of ions (e.g., protons) involved in coupling $\Delta\mu_H$. Thus, the phenomenological Onsager equations representing oxidative phosphorylation can be written as a 3×3 symmetric matrix in terms of conductances as follows:

$$J_O = L_{OO}A_O + L_{OH}\Delta\tilde{u}_H + L_{OP}A_P \quad (4)$$

$$J_H = L_{OH}A_O + L_{HH}\Delta\tilde{u}_H + L_{PH}A_P \quad (5)$$

$$J_P = L_{OP}A_O + L_{PH}\Delta\tilde{u}_H + L_{PP}A_P \quad (6)$$

The chemiosmotic theory postulates the absence of direct coupling between oxidation and ATP synthesis other than that mediated indirectly via the H^+ pumps through the protonmotive force. Hence, for chemiosmotic coupling, $L_{OP} = 0$. Thus the chemiosmotic theory fails to explain the phenomenon of respiratory control in oxidative phosphorylation. In the integrated model (Fig. 3), the binding of the cycled succinate anions to the respiratory enzyme complexes activates respiration from the mitochondrial matrix side. This direct coupling is shown by the horizontal line in the scheme of Fig. 3 and explains the control of the phosphorylation reaction on oxidation, and the phenomenon of respiratory control.

The classical chemiosmotic theory predicts that for any value of the respiration rate J_O there corresponds a unique value of $\Delta\mu_H$. Thus, an equal stimulation of respiration over state 4 respiration by either ADP or uncouplers is predicted to correspond to an equal reduction of $\Delta\mu_H$ by the theory (see Eq. (4) with $L_{OP} = 0$). However, we found in our experiments that stimulation of respiration with ADP was associated with a decrease in $\Delta\tilde{u}_H$ of only 5 mV, while the same stimulation of respiratory rate could only be achieved by a reduction of $\Delta\mu_H$ by as much as 30–35 mV when dinitrophenol was used as uncoupler [10,11,59,60]. These experimental observations are incompatible with the chemiosmotic theory but are readily explained by the integrated model of Fig. 3.

The above experimental observations are accommodated by the scheme of Fig. 3 because in addition to the indirect coupling mediated by the so-called protonmotive force of chemiosmosis, the binding to the redox enzyme complexes in the respiratory chain of succinate ions translocated through the ATP synthase along with protons initiates a direct effect on respiration. This direct interaction does not require the cycling of protons across bulk aqueous phases.

The direct effect discussed above can be modeled adequately by use of a finite positive value of L_{OP} in Nath's two-ion theory of energy coupling [20]. The author has recently completed evaluation of the six independent Onsager coefficients in Eqs. (4)–(6) from experimental data, thereby enabling a more complete description of oxidative phosphorylation, and a superior analysis of the optimality functions for the coupled nonequilibrium processes.

Declaration of Competing Interest

The author declares no conflict of interests.

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