

In preparation

2017-09-04; Version 32

Full-length review

The protonmotive force and respiratory control:

Building blocks of mitochondrial physiology

Part 1.

MitoEAGLE Working Group

Corresponding author: E. Gnaiger

Contributing co-authors (*alphabetical, to be extended*):

B. Ahn, M.G. Alves, D.A. Beard, D. Ben-Shachar, S. Breton, G.C. Brown, G.R. Buettner, A. Bumsoo, Z. Cervincova, A.J. Chicco, P.M. Coen, J.L. Collins, L. Crisóstomo, M.S. Davis, T. Dias, C. Doerrier, D.A. Fell, A. Filipovska, P.M. Garcia-Roves, L.F. Garcia-Souza, B.H. Goodpaster, D.K. Harrison, K.T. Hellgren, C.L. Hoppel, J. Iglesias-Gonzalez, B.A. Irving, S. Iyer, P. Jansen-Dürr, T. Käämbre, D.A. Kane, T. Komlodi, V. Laner, H.K. Lee, H. Lemieux, M. Makrečka-Kuka, A.T. Meszaros, N. Moiso, A. Molina, D. Montaigne, A.L. Moore, A.J. Murray, R.K. Porter, K. Nozickova, P.F. Oliveira, P.J. Oliveira, C.M. Palmeira, P.X. Petit, K. Renner-Sattler, J. Rohlena, D. Salvadego, L.A. Sazanov, M. Scheibye-Knudsen, G.R. Scott, D. Singer, O. Sobotka, M. Spinazzi, R. Stocker, M. Tanaka, B. Tandler, B. Velika, A.E.

Vercesi, Y.H. Wei, M.R. Wieckowski, A. Zorzano

Supporting co-authors (*alphabetical*):

O. Arandarčikaitė, B.M. Bakker, J. Batista Ferreira, P. Bernardi, H.E. Boetker, V. Borutaitė, R.A. Brown, E. Calabria, J.A. Calbet, E. Carvalho, B. Chaurasia, E. Clementi, A. Collin-

Chenot, A.M. Das, C. De Palma, G. Distefano, H. Dubouchaud, M. Duchen, W.J. Durham, J. Ehinger, E. Elmer, M. Fornaro, Z. Gan, K.D. Garlid, A. Garten, M.L. Genova, H. Gonzalo, C.W. Gourlay, C. Granata, J. Haendeler, J. Han, S.C. Hand, P. Hernansanz, A.J. Hickey, N.R. Jespersen, H. Kainulainen, G. Keppner, A.V. Khamoui, M. Klingenspor, W.J.H. Koopman, A.J. Kowaltowski, A. Krajcova, R. Kumar Jha, Labieniec-Watala M, G. Lenaz, E. Lucchinetti, L.A. MacMillan-Crow, M. Markova, M.A. Menze, A. Methner, J. Muntané, D.M. Muntean, J. Neuzil, S Newsom, D. O'Gorman, M.T. Oliveira, Z. Orynbayeva, Y.K. Pak, H.H. Patel, D. Pesta, S. Pirkmajer, E.V. Prochownik, T. Pulinilkunnil, MM Robinson, E.R. Ropelle, H.B. Rossiter, K. Salin, C. Sandi, J.M. Schilling, K. Siewiera, A.M. Silber, R. Skolik, B.T. Smenes, F.A.A. Soares, I. Sokolova, V.K. Sonkar, P. Stankova, Z. Sumbalova, R.H. Swerdlow, I. Szabo, J.P. Thyfault, A. Towheed, L. Tretter, K.J. Tronstad, A. Vieyra, D. Votion, C. Watala, R.C.I. Wust, K. Zaugg, M. Zaugg

Updates:

[http://www.mitoeagle.org/index.php/The_protonmotive_force_and_respiratory_control:
Building_blocks_of_mitochondrial_physiology](http://www.mitoeagle.org/index.php/The_protonmotive_force_and_respiratory_control:Building_blocks_of_mitochondrial_physiology)

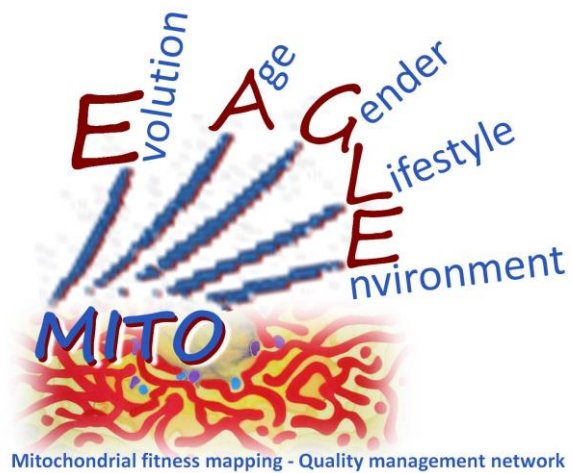
Correspondence: E. Gnaiger

Department of Visceral, Transplant and Thoracic Surgery, D. Swarovski Research Laboratory, Medical University of Innsbruck, Innrain 66/4, A-6020 Innsbruck, Austria

Email: erich.gnaiger@i-med.ac.at

Tel: +43 512 566796, Fax: +43 512 566796 20

This manuscript on 'Mitochondrial respiratory control' is in preparation as a position statement in the frame of COST Action CA15203 MitoEAGLE. Up to now, the list of co-authors is mainly based on previous MitoEAGLE Working Group Meetings. In the **bottom-up** spirit of COST, this is an open invitation to scientists and students to join as co-authors, to provide a balanced view on mitochondrial respiratory control, a fundamental introductory presentation of the concept of the protonmotive force, and a critical discussion on reporting mitochondrial respiration in terms of metabolic flows and fluxes. We plan a series of follow-up publications by the MitoEAGLE Terminology Group, to increase the scope of consensus-oriented recommendations and facilitate global communication and collaboration.



Mitochondrial fitness mapping - Quality management network

It would be great to receive your comments and suggestions by **2017-Sep-18**, particularly if you are an **early career investigator adding an open future-oriented perspective**, or an **established scientist providing a balanced historical basis**. Your critical input into the quality of the manuscript will be most welcome, improving our aims to be educational, general, consensus-oriented, and practically helpful for students working in mitochondrial respiratory physiology.

Please feel free to focus on a particular section in terms of direct input and references, while evaluating the entire scope of the manuscript from the perspective of your expertise.

We organize a MitoEAGLE session linked to our series of reviews at the MiPconference Nov 2017 in Hradec Kralove in close association with the MiPsociety (where you hopefully will attend) and at EBEC 2018 in Budapest.

» http://www.mitoeagle.org/index.php/MiP2017_Hradec_Kralove_CZ

I thank you in advance for your feedback.

With best wishes,

Erich Gnaiger

Chair Mitochondrial Physiology Society - <http://www.mitophysiology.org>

Chair COST Action MitoEAGLE - <http://www.mitoeagle.org>

Medical University of Innsbruck, Austria

Abstract

Clarity of concepts and consistency of nomenclature are trademarks of the quality of a research field across its specializations, facilitating transdisciplinary communication and education. As research and knowledge on mitochondrial physiology expand, the necessity for harmonization of nomenclature on mitochondrial respiratory states and rates has become apparent. Peter Mitchell's concept of the protonmotive force establishes the link between the electric and chemical components of energy transformation and coupling in oxidative phosphorylation. This unifying concept provides the framework for developing a consistent terminology on mitochondrial physiology and bioenergetics. We follow IUPAC guidelines on general terms of physical chemistry, extended by concepts of open systems and irreversible thermodynamics. We align the nomenclature of classical bioenergetics on respiratory states with a concept-driven constructive terminology to address the meaning of each respiratory state. Hence we focus primarily on the conceptual 'why' along with clarification of the experimental 'how'. The capacity of *oxidative phosphorylation*, OXPPOS, provides diagnostic reference values and is, therefore, measured at kinetically saturating concentrations of ADP, inorganic phosphate and fuel substrates. The contribution of *intrinsically uncoupled* oxygen consumption is most easily studied by arresting phosphorylation, when oxygen consumption compensates mainly for the proton leak, and the corresponding states are collectively classified as LEAK states. The *oxidative* capacity of the electron transfer system, ETS, reveals the limitation of OXPPOS capacity mediated by the *phosphorylation* system. Experimental standards for evaluation of respiratory coupling states must be followed for the development of databases of mitochondrial respiratory function in diverse physiological and experimental conditions and organisms.

Keywords: Mitochondrial respiratory control, coupling control; mitochondrial preparations, protonmotive force, chemiosmotic theory, oxidative phosphorylation, efficiency; electron transfer system, ETS; proton leak, LEAK; residual oxygen consumption, ROX; State 2, State 3, State 4.

Contents

1. Introduction

2. Fundamental respiratory coupling states in mitochondrial preparations

2.1. Definitions

Mitochondrial preparations

Phosphorylation, »P

Control and regulation

2.2. Classical terminology for isolated mitochondria

States 1-5

2.3. Three coupling states of mitochondrial preparations and residual oxygen consumption

OXPPOS, ETS, LEAK, ROX

3. States and rates

3.1. Respiratory states and respiratory rates

3.2. The steady-state and protonmotive force

Protonmotive force, Δp_{H^+}

Faraday constant, F

Electrical part of the protonmotive force, eI

Chemical diffusion (or dislocation) part of the protonmotive force, d

3.3. Forces and flows in physics and irreversible thermodynamics

Vectorial and scalar forces, and fluxes

Coupled versus bound processes

Coupling, efficiency, and power

3.4. Normalization: flows and fluxes

Extensive quantities

Size-specific quantities

Molar quantities

Flow per system, I

Size-specific flux, J

Flux per volume of the instrumental system, J_V

Sample concentration C_{mX} and C_{NX}

Mitochondrial concentration, C_{mte} , and mitochondrial markers

Mass-specific flux, J_{mX,O_2}

Mitochondria-specific flux, J_{mte,O_2}

Flow per sample entity, I_{X,O_2}

3.5. Conversion: oxygen, protons, ATP

4. Conclusions

- * Does the public expect that biologists understand Darwin's theory of evolution?
- * Do students expect that researchers of bioenergetics can explain Mitchell's theory of chemiosmotic energy transformation?

Mitochondria, mt: (Greek mitos: thread; chondros: granule) are organelles contained within eukaryotic cells with a double membrane separating the intermembrane space and the matrix with tubular or disk-shaped cristae. Mitochondria were described for the first time in 1857 by Rudolph Albert von Kölliker as granular structures – „sarkosomes”. 19 years later (1886) Richard Altman called them „bioblasts” (published in 1894). The word “Mitochondrium” was introduced by Carl Benda (1898). Mitochondria are the structural and functional elemental units of cell respiration. Mitochondria are the oxygen consuming electrochemical generators, where in the process of oxidative phosphorylation (OXPHOS) the reduction of O₂ is electrochemically coupled to conservation of energy in the form of ATP. In addition to the OXPHOS system, these powerhouses of the cell contain the cytochrome system and ATP synthase or alternative oxidases, ion transporters including proton pumps, mitochondrial kinases related to energy transfer pathways, the enzymes of the tricarboxylic acid cycle with several dehydrogenases, apoptotic proteins, and the fatty acid oxidation enzymes. The mitochondrial proteome comprises more than 1200 proteins (Mitocharta), mostly encoded by nuclear DNA (nDNA), with a variety of functions, many of which are still under investigation. Mitochondria maintain their own genetic material known as mitochondrial DNA (mtDNA) that is regulated by nuclear-encoded mitochondrially targeted proteins. There is a constant crosstalk between mitochondria and the other cellular components at the transcriptional or post-translational level as well as through cell signalling in response to varying energy demands. Mitochondrial morphology can also change in response to varying energy demands or external stimuli via processes known as fusion and fission through which mitochondria can communicate within a network. Abbreviation: mt, as generally used in

mtDNA. Mitochondrion is singular and mitochondria is plural. The bioblasts of Richard Altmann (1894) are not only the mitochondria as presently defined, but include symbiotic and free-living bacteria.

‘For the physiologist, mitochondria afforded the first opportunity for an experimental approach to structure-function relationships, in particular those involved in active transport, vectorial metabolism, and metabolic control mechanisms on a subcellular level’ (Ernster and Schatz 1981).

1. Introduction

Every study of mitochondrial function and disease is faced with **evolution, age, gender, lifestyle and environment (EAGLE)** as essential background conditions characterizing the individual patient or subject, cohort, species, tissue and to some extent even cell line. As a large and highly coordinated group of laboratories and researchers, the global MitoEAGLE network is uniquely poised to generate the necessary scale, type, and quality of consistent data sets and conditions to address this intrinsic complexity. The mission of the MitoEAGLE network aims at developing harmonized experimental protocols and implementing a quality control and data management system to interrelate results obtained in different studies and to generate a rigorously monitored database focused on mitochondrial respiratory function.

Reliability and comparability of quantitative results depends on the accuracy of measurement under well-defined conditions. A conceptually meaningful framework also is required to relate the results of experiments carried out by different research groups. With an emphasis on quality of research, data gathered can be useful far beyond the specific question of an experiment. Vague or ambiguous terminology can lead to confusion and may relegate valuable signals to wasteful noise. For this reason, measured values must be expressed in standardized units for each parameter used to define mitochondrial respiratory control. Standardization of nomenclature and technical jargon is essential to improve the awareness of

the intricate meaning of divergent scientific vocabulary. The MitoEAGLE Terminology Group aims at accomplishing the ambitious goal of harmonizing, unifying and thus simplifying the terminology in the field of mitochondrial physiology. A focus on coupling states in mitochondrial preparations is a first step in the attempt to generate a harmonized and conceptually oriented nomenclature in bioenergetics and mitochondrial physiology. Comparison with coupling states of intact cells (Wagner *et al.* 2011) and respiratory control by fuel substrates and specific inhibitors of respiratory enzymes (Gnaiger 2009; 2014) will be reviewed in subsequent recommendations.

2. Fundamental respiratory coupling states in mitochondrial preparations

‘Every professional group develops its own technical jargon for talking about matters of critical concern ... People who know a word can share that idea with other members of their group, and a shared vocabulary is part of the glue that holds people together and allows them to create a shared culture’ (Miller 1991).

2.1. Definitions

Mitochondrial preparations are defined as tissue or cellular preparations in which the plasma membrane is either removed (isolated mitochondria), or mechanically and/or chemically permeabilized (tissue homogenate, permeabilized fibres, permeabilized cells), while the functional integrity and, to a large extent, the structure of mitochondria are maintained. The intact, non-permeabilized plasma membrane prevents the free passage of many water-soluble mitochondrial substrates and inhibitors, limiting the scope of investigations into mitochondrial respiratory function, whilst the cycling of ATP/ADP in intact cells prohibits an analysis of the control exerted by ADP on respiration.

Respiratory control is monitored in a mitochondrial preparation under experimental conditions defined as respiratory states. Coupling states in mitochondrial preparations depend on an exogenous supply of fuel substrates and oxygen to support the electron transfer system,

ETS (**Fig. 1**). When phosphorylation of ADP to ATP is stimulated or depressed, an increase or decrease is observed of electron flow linked to oxygen consumption in ‘controlled’ coupling states. Alternatively, coupling of electron transfer with phosphorylation is disengaged by uncouplers, functioning like a clutch in a mechanical system. The corresponding respiratory state is characterized by high levels of oxygen consumption without control by phosphorylation (‘uncontrolled state’). Such uncoupling is different from switching to mitochondrial pathways that involve less than three coupling sites (Complexes CI, CIII and CIV), bypassing CI through multiple electron entries into the Q-junction (**Fig. 1**). A bypass of CIV is provided by alternative oxidases, which reduce oxygen without proton translocation. Reprogramming mitochondrial pathways may be considered as a switch of gears (stoichiometry) rather than uncoupling (loosening the stoichiometry).

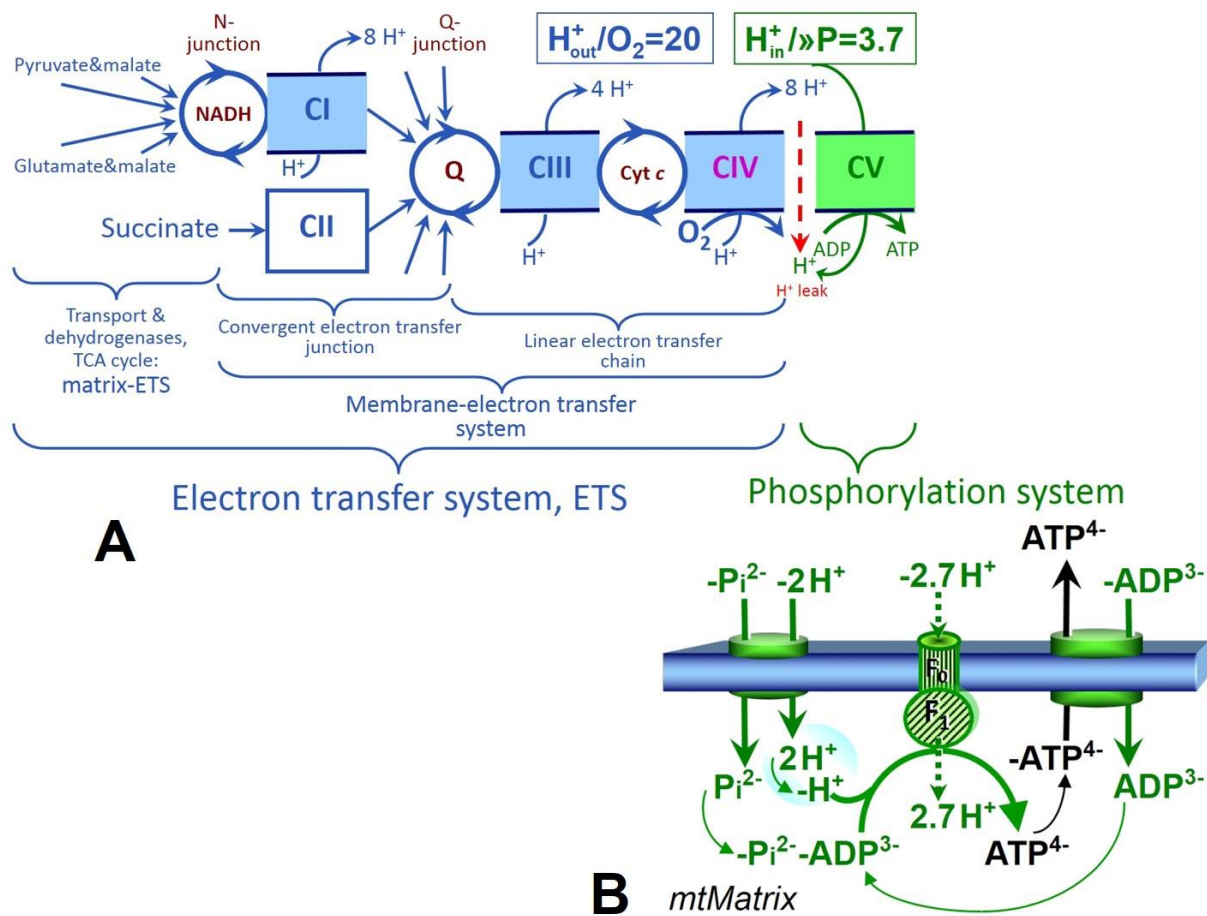


Fig. 1. The mitochondrial respiratory system. In oxidative phosphorylation, the electron transfer system, ETS is coupled to the phosphorylation system. (A) ETS (; to be updated by L

Sazalov) Multiple convergent electron transfer pathways are shown from NADH and succinate and additional arrows (electron transferring flavoprotein, glycerophosphate dehydrogenase, and others). H^+_{out}/O_2 is the ratio of outward proton flux from the matrix space to catabolic O_2 flux in the NADH-linked pathway. $H^+_{in}/\gg P$ is the ratio of inward proton flux from the inter-membrane space to the endergonic flux of phosphorylation of ADP to ATP. Due to proton leak and slip these are not fixed stoichiometries. **(B)** The $H^+_{in}/\gg P$ stoichiometry is the sum of the coupling stoichiometry in the ATP synthase reaction (-2.7 H^+ from the intermembrane space, 2.7 H^+ to the matrix) and the proton balance in the translocation of ADP^{2-} , ATP^{3-} and P_i^{2-} . See Eqs. 11 and 12 for further explanation. Modified from (A) Lemieux *et al.* (2017) and (B) Gnaiger (2014).

Phosphorylation, $\gg P$: *Phosphorylation* in the context of OXPHOS is defined as phosphorylation of ADP to ATP. On the other hand, the term phosphorylation is used generally in many different contexts, *e.g.* protein phosphorylation. This justifies consideration of a symbol more discriminative than P as used in the P/O ratio (phosphate to atomic oxygen ratio), where P indicates phosphorylation of ADP to ATP or GDP to GTP. We propose the symbol $\gg P$ for the endergonic direction of phosphorylation coupled to catabolic reactions, and likewise the symbol $\ll P$ for the corresponding exergonic hydrolysis (upwards and downwards arrows, respectively, in **Fig. 2**). ATP synthase is the most important enzyme complex of the phosphorylation system (**Fig. 1B**); however $\gg P$ may also involve substrate-level phosphorylation as part of the tricarboxylic acid cycle (succinyl-CoA ligase), and phosphorylation of ADP catalysed by phosphoenylpyruvate carboxykinase, adenylate kinase, creatine kinase, hexokinase and nucleoside diphosphate kinase (NDPK). Kinase cycles are involved in intracellular energy transfer and signal transduction for regulation of energy flux. In isolated mammalian mitochondria ATP production catalysed by adenylate kinase, $2ADP \leftrightarrow$

ATP + AMP, proceeds without fuel substrates in the presence of ADP (Komlódi and Tretter 2017).

Control and regulation: The terms metabolic *control* and *regulation* are frequently used synonymously, but are distinguished in metabolic control analysis: ‘*We could understand the regulation as the mechanism that occurs when a system maintains some variable constant over time, in spite of fluctuations in external conditions (homeostasis of the internal state). On the other hand, metabolic control is the power to change the state of the metabolism in response to an external signal*’ (Fell 1997). Respiratory control may be exerted by changing experimental variables that exert an influence on: (1) ATP demand; (2) fuel substrate, pathway competition and oxygen availability (*e.g.* starvation and hypoxia); (3) the protonmotive force, redox states, flux-force relationships, coupling and efficiency; (4) mitochondrial enzyme activities and allosteric regulation by adenylates, phosphorylation of regulatory enzymes, Ca^{2+} and other ions including H^+ ; (5) inhibitors (*e.g.* nitric oxide or intermediary metabolites, such as oxaloacetate); (6) metabolic channeling by supercomplexes; (7) enzyme content, concentrations of cofactors and conserved moieties (such as adenylates, NAD^+/NADH , coenzyme Q, cytochrome *c*); and (8) mitochondrial density (enzyme concentrations and membrane area) and morphology (fission and fusion). **E**volutionary or acquired differences in the genetic basis of mitochondrial function (or dysfunction) between subjects and gene therapy; **A**ge; **G**ender and hormone concentrations; **L**ife style including exercise and nutrition; and **E**nvironmental including toxicological and pharmacological factors (EAGLE) exert an influence on all control mechanisms listed above (for reviews, see Brown 1992; Gnaiger 1993a, 2009; 2014; Morrow *et al.* 2017).

Respiratory control and response: There is a difference between control by a fixed component of a metabolic system (*e.g.* the ETS or phosphorylation system; **Fig. 1**) and the response to an experimental variable (*e.g.* fuel substrate or ADP). Whilst lack of control by a metabolic module (*e.g.* phosphorylation system) does mean that there will be no response to a

variable activating it (*e.g.* [ADP]), the reverse is not true; *i.e.*, lack of response to [ADP] does not exclude phosphorylation having some degree of control. The degree of control of a component of the OXPHOS system on an output variable of the system, such as oxygen flux, will in general be different from the degree of control on other outputs, such as phosphorylation flux, cytochrome redox states, protonmotive force, phosphorylation potential, proton leak flux. As such, it is necessary to be specific about which output is being considered. Respiratory control is insufficiently specific in the context of specific interpretations (Fell 1997).

2.2. Classical terminology for isolated mitochondria

‘When a code is familiar enough, it ceases appearing like a code; one forgets that there is a decoding mechanism. The message is identical with its meaning’
(Hofstadter 1979).

Chance and Williams (1955; 1956) introduced five classical states of mitochondrial respiration and cytochrome redox states. **Table 1** shows a protocol with isolated mitochondria in a closed respirometric chamber, defining a sequence of respiratory states.

State 1 is obtained after addition of isolated mitochondria to air-saturated isoosmotic/isotonic respiration medium containing inorganic phosphate, but no adenylates (*i.e.* AMP, ADP, ATP) and no fuel substrates.

State 2 is induced by addition of a high concentration of ADP (typically 100 to 300 μM), which stimulates respiration transiently on the basis of endogenous fuel substrates and phosphorylates only a small portion of the added ADP. State 2 is then obtained at a low respiratory activity limited by zero endogenous fuel substrate availability (**Table 1**). If addition of specific inhibitors of respiratory complexes, such as rotenone, do not cause a further decline of oxygen consumption, State 2 is equivalent to residual oxygen consumption (See below). If inhibition is observed, undefined endogenous fuel

substrates are a confounding factor of pathway control by externally added substrates. More recently, a re-definition of State 2 caused confusion, considering an alternative protocol when fuel substrate is added in the second step without ADP (LEAK state, see below), followed by addition of ADP (Nicholls and Ferguson 2013).

State 3 is the state stimulated by addition of fuel substrates while the ADP concentration is still high (**Table 1**) and supports coupled energy transformation through oxidative phosphorylation. 'High ADP' is a concentration of ADP specifically selected to allow the measurement of State 3 to State 4 transitions of isolated mitochondria in a closed respirometric system. A repeated ADP titration re-establishes State 3 at 'high ADP'. Starting at oxygen concentrations near air-saturation (ca. 200 $\mu\text{M O}_2$), the total ADP concentration added must be low enough (typically 100 to 300 μM) to allow phosphorylation to ATP at a coupled oxygen consumption that does not lead to oxygen depletion during the transition to State 4. In contrast, kinetically saturating ADP concentrations usually are an order of magnitude higher than 'high ADP', *e.g.* 2.5 mM in isolated mitochondria.

State 4 is reached only if the mitochondrial preparation is intact and well-coupled. Depletion of ADP by phosphorylation to ATP will then lead to a decline in oxygen uptake in the transition from State 3 to State 4. Under these conditions a maximum protonmotive force and high ATP/ADP ratio are maintained. State 4 respiration reflects intrinsic proton leak and contaminating ATP hydrolysis activity.

State 5 is a state obtained after exhaustion of oxygen in a closed respirometric chamber. Diffusion of oxygen from the surroundings into the aqueous solution may be a confounding factor preventing complete anoxia (Gnaiger 2001).

Table 1. Metabolic states of mitochondria (after Chance and Williams, 1956).

State	[O ₂]	[ADP]	[Substrate]	Respiration rate	Rate-limiting substance
1	>0	Low	Low	Slow	ADP
2	>0	High	~0	Slow	Substrate
3	>0	High	High	Fast	Respiratory chain
4	>0	Low	High	Slow	ADP
5	0	High	High	0	Oxygen

2.3. Three coupling states of mitochondrial preparations and residual oxygen consumption

To extend the classical nomenclature (differential: States 1 to 5) by a concept-driven terminology that incorporates explicit information on the nature of the respiratory states, the terminology must be general and not restricted to any particular experimental protocol or mitochondrial preparation (Gnaiger 2009). In the following section, the new concept driven terminology is explained and coupling states are defined (**Table 2**). Coupling states of mitochondrial preparations can be compared in any mitochondrial pathway control state, *i.e.* keeping fuel substrates and ETS inhibitors constant while varying adenylate concentrations, inhibitors of the phosphorylation system and performing uncoupler titrations (**Figs. 2 to 5**).

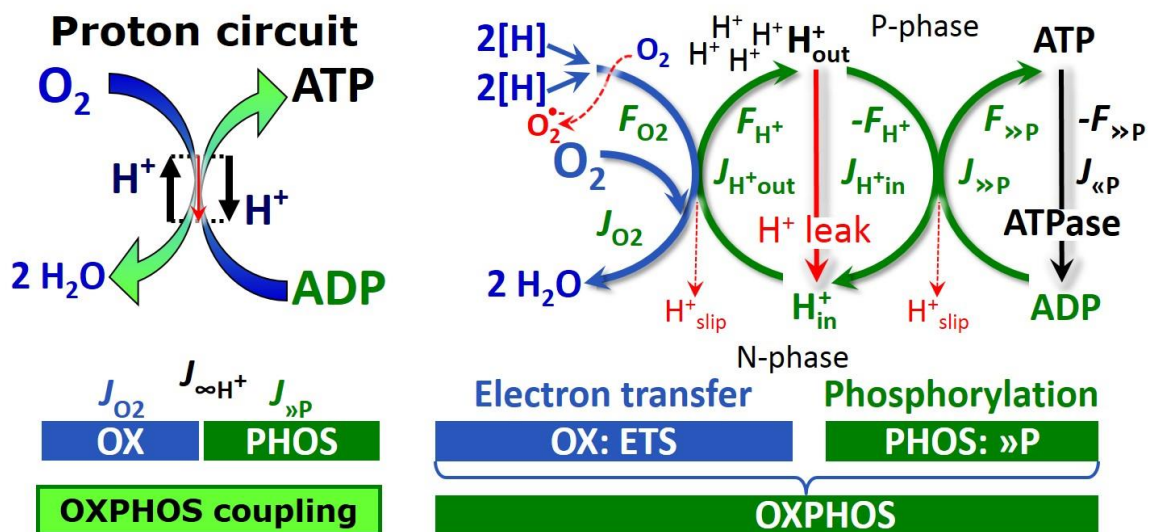


Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). Oxygen flux, J_{O_2} , is coupled to the phosphorylation of ADP to ATP, $J_{»P}$, by the proton pumps of the

electron transfer system, ETS, pushing the outward proton flux, $J_{H^{+}out}$, and generating the output protonmotive force, $F_{H^{+}}$. ATP synthase is driven by the input protonmotive force, $-F_{H^{+}}$, and inward proton flux, $J_{H^{+}in}$, to phosphorylate ADP to ATP. $2[H]$ indicates the reduced hydrogen equivalents of fuel substrates that provide the chemical input force, F_{O_2} [kJ/mol O_2], of the reaction with oxygen (molar Gibbs energy of reaction), typically in the range of -460 to -480 kJ/mol. The output force is given by the phosphorylation potential difference, $F_{\gg P}$ [kJ/mol ADP phosphorylated to ATP], which varies *in vivo* in a range of about 48 to 62 kJ/mol under physiological conditions. Proton turnover, $J_{\infty H^{+}}$, and ATP turnover, $J_{\infty P}$, proceed in the steady state at constant $F_{H^{+}}$, when $J_{\infty H^{+}} = J_{H^{+}out} = J_{H^{+}in}$, and at constant $F_{\gg P}$, when $J_{\infty P} = J_{\gg P} = J_{\ll P}$. $J_{\gg P}/J_{O_2}$ ($\gg P/O_2$) is two times the ‘P/O’ ratio of classical bioenergetics. The effective $\gg P/O_2$ ratio is diminished by: (1) the proton leak across the inner mitochondrial membrane from low pH in the P-phase to high pH in the N-phase (P, positive; N, negative); (2) cycling of other cations; (3) proton slip of the proton pumps when effectively a proton is not pumped; and (4) electron leak in the univalent reduction of oxygen (O_2 ; dioxygen) to superoxide anion radical ($O_2^{\bullet -}$). Modified from Gnaiger (2014).

Table 2. Coupling states and ROX in mitochondrial preparations.

State	Respiration	Δp_{H^+}	Induced by:	Limited by:
LEAK ¹	<i>L</i> ; low, proton leak-dependent respiration	Max.	Kinetically saturating [substrate] and [O ₂] (<i>I</i>) without ADP, <i>L_N</i> ; (2) with max. ATP/ADP ratio (State 4); or (3) with full inhibition of the ADP phosphorylation system (State 4o)	Proton leak
OXPHOS	<i>P</i> ; high, ADP-stimulated respiration	High	Kinetically saturating [substrate], [O ₂], [ADP], and [P _i] (State 3, possibly without kinetic saturation of ADP)	Phosphorylation system or electron transfer system
ETS	<i>E</i> ; maximal, noncoupled respiration	Min.	Kinetically saturating [substrate] and [O ₂] at optimal uncoupler concentration for maximum oxygen flux (State 3u)	Electron transfer system
ROX	<i>Rox</i> , Minimum, residual (non-ETS) oxygen consumption	0	Full inhibition of ETS or absence of substrates	Non-ETS oxidation reactions

OXPHOS state (Fig. 3): The respiratory state with kinetically saturating concentrations of O₂, and phosphorylation substrates, and zero uncoupler, to provide an estimate of the maximal capacity of OXPHOS in any given

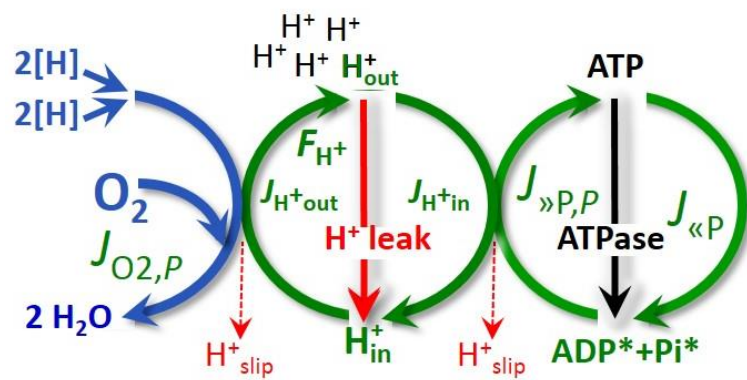


Fig. 3. OXPHOS state: Phosphorylation, $J_{\gg P}$, is stimulated by kinetically saturating [ADP]* and inorganic phosphate, [P_i]*, and is supported by a high protonmotive force, F_{H^+} . O₂ flux, $J_{O_2,P}$, is highly coupled at a maximum $\gg P/O_2$ ratio, $J_{\gg P,P}/J_{O_2,P}$ (See also Fig. 2).

pathway control state. Respiratory capacities at kinetically saturating substrate concentrations provide reference values or upper limits of performance, aiming at the generation of data sets for comparative purposes. Any effects of substrate kinetics are thus separated from reporting actual mitochondrial capacities, against which physiological activities can be evaluated.

As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated mitochondria (Gnaiger 2001; Puchowicz *et al.* 2004); higher ADP concentrations are required, particularly in permeabilized muscle fibres, to overcome limitations by diffusion and by the tubulin-regulated conductance of the outer mitochondrial membrane (Rostovtseva *et al.* 2008). In permeabilized muscle fibre bundles of high respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks *et al.* 1998). This implies that >90% saturation is reached only at >5 mM ADP. Similar ADP concentrations are also required for accurate determination of OXPHOS capacity in human clinical cancer samples and permeabilized cells.

ETS state (Fig. 4): The ETS state is defined as the *noncoupled* state with kinetically saturating concentrations of O_2 , respiratory substrate and optimum *exogenous* uncoupler concentration for maximum O_2 flux, as an estimate of oxidative

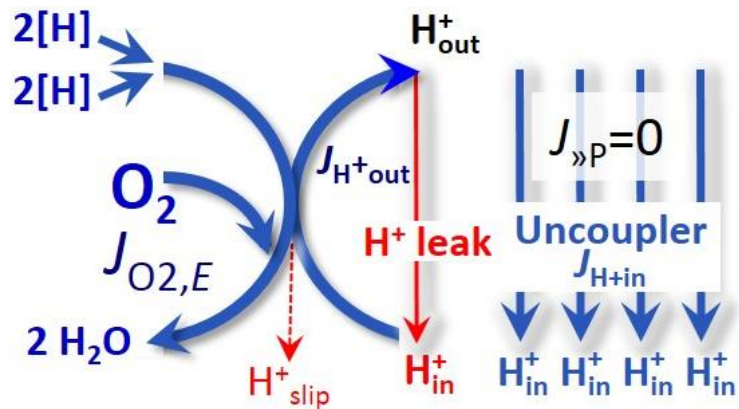


Fig. 4. ETS state: Noncoupled respiration, $J_{O_2,E}$, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, $J_{\gg P}=0$ (See also Fig. 2).

ETS capacity. As a consequence of the nearly collapsed protonmotive force, the driving force for phosphorylation is missing and $J_{\gg P}=0$. The abbreviation State 3u is frequently

used in bioenergetics, to indicate the state of maximum respiration without sufficient emphasis on the fundamental difference between OXPHOS capacity (*well coupled* with an *endogenous* uncoupled component) and ETS capacity (*noncoupled*).

LEAK state (Fig. 5): A state of mitochondrial respiration when O_2 flux mainly compensates for the proton leak in the absence of ATP synthesis, at kinetically saturating concentrations of O_2 and respiratory substrates. LEAK respiration is measured to obtain an indirect estimate

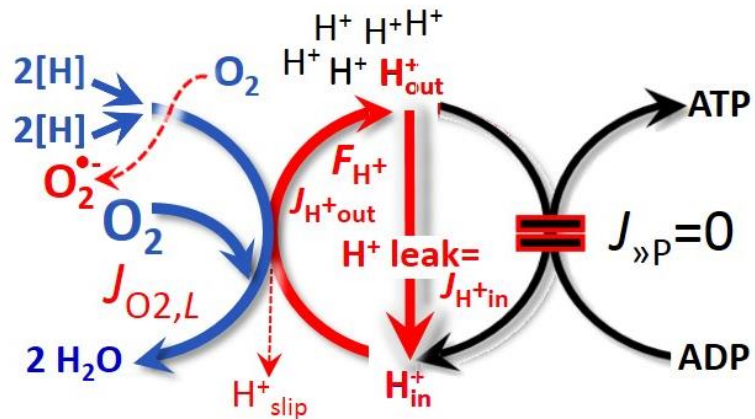


Fig. 5. LEAK state: Phosphorylation is arrested, $J_{\gg P}=0$, and oxygen flux, $J_{O_2,L}$, is controlled mainly by the proton leak, which equals J_{H^+in} , at maximum protonmotive force, F_{H^+} (See also Fig. 2).

of *intrinsic uncoupling* without addition of any experimental uncoupler: (1) in the absence of adenylates; (2) after depletion of ADP at maximum ATP/ADP ratio; or (3) after inhibition of the phosphorylation system by inhibitors of ATP synthase, such as oligomycin (frequently called State 4o), or adenylate nucleotide translocase, such as carboxyatractyloside. Oxygen consumption in State 4 is an overestimation of LEAK respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP, $J_{\gg P}$, which stimulates respiration coupled to phosphorylation, $J_{\gg P}>0$. This can be tested by inhibition of the phosphorylation system using oligomycin, ensuring that $J_{\gg P}=0$.

Proton leak: Proton leak is the *uncoupled* process in which protons are translocated across the inner mitochondrial membrane in the dissipative direction of the downhill protonmotive force without coupling to phosphorylation (**Fig. 5**). The proton leak flux

depends on the protonmotive force, is a property of the inner mitochondrial membrane, and may be physiologically controlled. Mitochondrial injuries may lead to *dyscoupling* as a pathological or toxicological cause of *uncoupled* respiration, e.g. as a consequence of opening the permeability transition pore. Dyscoupled respiration is distinguished from the experimentally induced *noncoupled* respiration in the ETS state. Under physiological conditions the proton leak is the dominant contributor to the overall leak current.

Proton slip: Proton slip is the *decoupled* process in which protons are only partially translocated by a proton pump of the ETS and slip back to the original compartment (Dufour *et al.* 1996). Proton slip can also happen at the ATP-synthase, in which case the proton slips downhill across the membrane to the matrix without contributing to ATP synthesis. In each case proton slip is a property of the proton pump and depends on the turnover rate of the pump.

Cation cycling: Proton leak is a leak current of protons. There can be other cation contributors to leak current including calcium and probably magnesium. Calcium current is balanced by Na/Ca exchange, which is balanced by Na/H exchange or K/H exchange. This is another effective uncoupling mechanism different from proton leak and slip.

Uncoupling protein: Uncoupling mediated by uncoupling protein 1 is physiologically controlled, *e.g.* in brown adipose tissue.

Table 3. Distinction of terms related to coupling.

Term	Respiration	$\gg P/O_2$	Note
Fully coupled	$P - L$	Max.	OXPPOS capacity corrected for LEAK respiration (Fig. 6)
Coupled	P	High	Phosphorylating respiration with a variable component of intrinsic LEAK respiration (Fig. 3)
Uncoupled, decoupled	L	0	Nonphosphorylating respiration without added protonophore (Fig. 5)
Noncoupled	E	0	Nonphosphorylating respiration stimulated to maximum flux at optimum uncoupler concentration (Fig. 4)
Dyscoupled	P	Low	Pathologically increased uncoupling, mitochondrial dysfunction

Small differences of terms (*e.g.* uncoupled, noncoupled) are easily overlooked and perceived as the same. Even with an attempt at rigorous definition, the common use of such terms may remain vague (**Table 3**). Besides the three main coupling states of mitochondrial preparations, the following respiratory state is also relevant to assess respiratory function:

ROX: Residual oxygen consumption (ROX) is defined as O_2 consumption due to oxidative side reactions remaining after inhibition of the ETS. ROX is not a coupling state but represents a baseline that is used to correct mitochondrial respiration in defined coupling states. ROX is not necessarily equivalent to non-mitochondrial respiration, considering oxygen-consuming reactions in mitochondria not related to ETS, such as oxygen consumption in reactions catalyzed by monoamine oxidases. In the presence of O_2 , ROX is measured either in the absence of fuel substrates or after blocking the electron supply to cytochrome *c* oxidase and alternative oxidases.

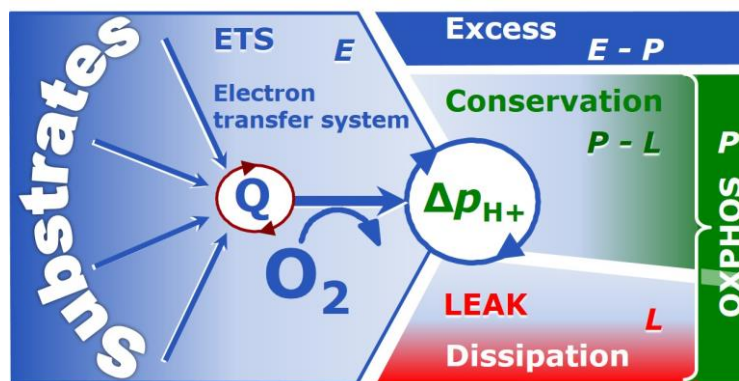
3. States and rates

3.1. Coupling states and respiratory rates

The protonmotive force is *high* in the OXPPOS state when it drives phosphorylation, *maximum* in the LEAK state of coupled mitochondria, driven by LEAK respiration at a

minimum back flux of protons to the matrix side, and *very low* in the ETS state when uncouplers short-circuit the proton cycle (**Table 2**).

Fig. 6. Four-compartmental model of oxidative phosphorylation with respiratory states (ETS, OXPPOS, LEAK) and corresponding rates (E , P , L).



Electron transfer system capacity, E , is partitioned into the dissipative LEAK respiration, L , partial conservation of the protonmotive exergy as the phosphorylation exergy in net OXPPOS capacity, $P-L$, and the excess capacity, $E-P$. Modified from Gnaiger (2014).

Fig. 6 summarizes the three coupling states, ETS, LEAK and OXPPOS, and puts them into a schematic context with the corresponding respiratory rates, abbreviated as E , L and P , respectively. This clarifies that E may exceed or be equal to P , but E cannot theoretically be lower than P . $E < P$ must be discounted as an artefact, which may be caused experimentally by: (1) using too low uncoupler concentrations; (2) using high uncoupler concentrations which inhibit the ETS (Gnaiger 2008); (3) high oligomycin concentrations applied for measurement of L before titrations of uncoupler, when oligomycin exerts an inhibitory effect on E ; or (4) loss of oxidative capacity during the time course of the respirometric assay with E measured subsequently to P (Gnaiger 2014). On the other hand, the excess ETS capacity is overestimated if non-saturating [ADP] or [P_i] (State 3) are used.

$E > P$ is observed in many types of mitochondria and depends on: (1) the excess ETS capacity pushing the phosphorylation system (**Fig. 1B**) to the limit of its *capacity of utilizing* Δp_{H^+} ; (2) the pathway control state with single or multiple electron input into the Q-junction

and involvement of three or fewer coupling sites determining the H^+_{out}/O_2 coupling stoichiometry (**Fig. 1A**); (3) the biochemical coupling efficiency expressed as $(E-L)/E$, since any increase of L causes an increase of P upwards to the limit of E . The excess $E-P$ capacity, $ExP=E-P$, therefore, provides a sensitive diagnostic indicator of specific injuries of the phosphorylation system, under conditions when E remains constant but P declines relative to controls (**Fig. 6**). Substrate cocktails supporting simultaneous convergent electron transfer to the Q-junction for reconstitution of TCA cycle function stimulate ETS capacity, and consequently increase the sensitivity of the ExP assay.

When subtracting L from P , the dissipative LEAK component in the OXPHOS state may be overestimated. This can be avoided by measurement of LEAK respiration in a state when the protonmotive force is titrated, *e.g.* by an ETS inhibitor, to the slightly lower value maintained in the OXPHOS state. Any turnover-dependent components of proton leak and slip, however, are underestimated under these conditions (Garlid *et al.* 1993). In general, it is inappropriate to use the term *ATP production* for the difference of oxygen consumption measured in states P and L . The difference $P-L$ is the upper limit of the part of OXPHOS capacity that is *free* (corrected for LEAK respiration) and is fully coupled to phosphorylation with a maximum mechanistic stoichiometry (**Fig. 6**).

3.2. The steady-state and protonmotive force

Mitochondria represent thermodynamically open systems functioning as biochemical reaction systems in non-equilibrium states. State variables (membrane potential difference; redox states) and metabolic fluxes (*rates*) are measured in defined mitochondrial respiratory states. Strictly, steady states can be obtained only in open systems, in which changes due to internal transformations (*e.g.* O_2 consumption) are instantaneously compensated by external flows (*e.g.* O_2 supply), such that oxygen concentration does not change in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored in closed systems satisfy the

criteria of pseudo-steady states for limited periods of time, when changes in the system (concentrations of O₂, fuel substrates, ADP, Pi, H⁺) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require buffers and kinetically saturating concentrations of substrates to be maintained, and thus depend on the kinetics of the processes under investigation.

Protonmotive force, Δp_{H^+} : The protonmotive force across the inner mitochondrial membrane (Mitchell and Moyle 1967),

$$\Delta p_{H^+} = \Delta \Psi + \Delta \mu_{H^+}/F \quad (\text{Eq. 1})$$

consists of an electric part, $\Delta \Psi$, which is the difference of charge (electric potential difference) and is not specific for H⁺, and of a chemical part, $\Delta \mu_{H^+}$, which is the chemical potential difference of H⁺, is proportional to the pH difference and incorporates the Faraday constant (**Table 4**).

Table 4. Protonmotive force matrix. Rows: Electric and chemical isomorph format (e and n). The Faraday constant, F , converts protonmotive force and flow from *isomorph format e* to n . Columns: The protonmotive force is the sum of *partial isomorph forces* F_{el} and F_{d,H^+} . In contrast to force (state), the conjugated flow (rate) cannot be partitioned.

State	Force	electric	+	chemical	Unit	Notes		
Protonmotive force, e	Δp_{H^+}	$= \Delta \Psi$	+	$\Delta \mu_{H^+}/F$	J/C	Eq. 1e		
Chemiosmotic potential, n	$\Delta \tilde{\mu}_{H^+}$	$= \Delta \Psi \cdot F$	+	$\Delta \mu_{H^+}$	J/mol	Eq. 1n		
State	Isomorph i	Force, $F_{H^+/i}$	=	eI	+	d,H⁺		
	Electric charge, e	$F_{H^+/e}$	=	$F_{el/e}$	+	$F_{d,H^+/e}$	J/C	Eq. 2e
	Amount of substance, n	$F_{H^+/n}$	=	$F_{el/n}$	+	$F_{d,H^+/n}$	J/mol	Eq. 2n
Rate	Isomorph i	Flow, $I_{H^+/i}$	=	e	or	n		
	Electric charge, e	$I_{H^+/e}$		$I_{H^+/e}$			C/s	
	Amount of substance, n	$I_{H^+/n}$				$I_{H^+/n}$	mol/s	

Eq. 1: The Faraday constant, F , is the product of elementary charge ($e=1.602177\cdot 10^{-19}\cdot\text{C}$) and the Avogadro (Loschmidt) constant ($N_A=6.022136\cdot 10^{23}\cdot\text{mol}^{-1}$), $F=eN_A=96,485.3$ C/mol. $\Delta\tilde{\mu}_{\text{H}^+}$ is the chemiosmotic potential difference. Eq. 1 is the classical representation of Eq. 2e.

Eq. 2: The signs depend on the definition of the compartmental direction of the translocation (**Fig. 2**). Flow x force = $I_{\text{H}^+/e}\cdot F_{\text{H}^+/e} = I_{\text{H}^+/n}\cdot F_{\text{H}^+/n} = \text{Power [J/s=W]}$.

Faraday constant, F : The Faraday constant yields the conversion between protonmotive force, $F_{\text{H}^+/e} \equiv \Delta p_{\text{H}^+}$ [J/C], expressed per *motive charge*, e [C], and protonmotive force or electrochemical (chemiosmotic) potential difference, $F_{\text{H}^+/n} \equiv \Delta\tilde{\mu}_{\text{H}^+} = \Delta p_{\text{H}^+}\cdot F$ [J/mol], expressed per *motive amount of protons*, n [mol]. Proton charge, e , and amount of substance, n , define the units for the isomorphic formats. Taken together, $F=eN_A$ [C/mol] converts protonmotive force and flow from isomorphic format e to n (**Table 4**, Eq. 2),

$$F_{\text{H}^+/n} = F_{\text{H}^+/e} \cdot eN_A \quad (\text{Eq. 3.1})$$

$$I_{\text{H}^+/n} = I_{\text{H}^+/e} / (eN_A) \quad (\text{Eq. 3.2})$$

In each case, the protonmotive force is expressed as the sum of two partial forces. The rather complicated symbols in Eq. 1 can be explained and visualized more easily by *isomorphic partial protonmotive forces*:

Electric part of the protonmotive force, el: (1) Isomorph e : $F_{\text{el}/e} \equiv \Delta\Psi$ is the electric part of the protonmotive force expressed in units joule per coulomb, *i.e.* volt [$\text{V}=\text{J}/\text{C}$], defined as partial Gibbs energy change per *motive elementary charge*, e [C], not specific for proton charge (**Table 4**, Eq. 2e).

(2) Isomorph n : $F_{\text{el}/n} \equiv \Delta\Psi\cdot F$ is the same quantity expressed in units joule per mole [J/mol], defined as partial Gibbs energy change per *motive amount of charge*, n [mol], not specific for proton charge (**Table 4**, Eq. 2n).

Chemical diffusion (translocation) part of the protonmotive force, d : (1) Isomorph n : $F_{d,H^+/n} \equiv \Delta\mu_{H^+}$ is the chemical part of the protonmotive force expressed in units joule per mole [J/mol], defined as partial Gibbs energy change per *motive amount of protons*, n [mol] (**Table 4**, Eq. 2*n*).

(2) Isomorph e : $F_{d,H^+/e} \equiv \Delta\mu_{H^+}/F$ is the chemical force expressed in units joule per coulomb [V], defined as partial Gibbs energy change per *motive amount of protons expressed in units of electric charge*, e [C], but specific for proton charge (**Table 4**, Eq. 2*e*).

Protonmotive means that protons are moved across the mitochondrial membrane at constant force, which is a measure of the potential for motion. Motion is relative and not absolute (Principle of Galilean Relativity), and likewise there is no absolute potential, but (isomorphic) forces are potential differences (equations in **Table 5**). A membrane potential difference, $F_{el/e}$, of -0.2 V (Eq. 4*e*) equals an electrochemical potential difference, $F_{el,H^+/n}$, of 19 kJ·mol⁻¹ H⁺_{out} (Eq. 4*n*). For a Δ pH of 1 unit, the chemical force, $F_{d,H^+/n}$, changes by 6 kJ·mol⁻¹ (Eq. 5*n*) and $F_{d,H^+/e}$ changes by 0.06 V (**Table 5**, Eq. 5*e*). Considering a driving force of -470 kJ·mol⁻¹ O₂ for oxidation, the thermodynamic limit of the H⁺_{out}/O₂ ratio is reached at a value of 470/19=24, compared with a mechanistic stoichiometry of 20 (**Fig. 1**).

Table 5. Force, flow, energy, and power.

Expression	Symbol	Definition	Unit	Notes
e : electric partial force	$F_{el/e}$	$F_{el/e} \equiv \Delta\Psi$	V	Eq. 4e
n : electric partial force	$F_{el/n}$	$\Delta\Psi \cdot F = 96.5 \cdot \Delta\Psi$	$\text{kJ} \cdot \text{mol}^{-1}$	Eq. 4n
e : chemical partial force at 37 °C	$F_{d,H+/e}$	$\Delta\mu_{H+}/F = -\ln(10) \cdot RT/F \cdot \Delta\text{pH}$ $= -0.06 \cdot \Delta\text{pH}$	V $\text{J} \cdot \text{C}^{-1}$	Eq. 5e
n : chemical partial force at 37 °C	$F_{d,H+/n}$	$\Delta\mu_{H+} = -\ln(10) \cdot RT \cdot \Delta\text{pH}$ $= -5.9 \cdot \Delta\text{pH}$	$\text{J} \cdot \text{mol}^{-1}$ $\text{kJ} \cdot \text{mol}^{-1}$	Eq. 5n
Force, isomorphic	F_{tr}	$F_{tr} = \partial G \cdot \partial_{tr} \zeta^{-1}$	$\text{J} \cdot \text{X}^{-1}$	Eq. 6
Flow, isomorphic	I_{tr}	$I_{tr} = d_{tr} \zeta \cdot dt^{-1}$	$\text{X} \cdot \text{s}^{-1}$	Eq. 7
n : Advancement n	$d_{tr} \zeta_{H+/n}$	$d_{tr} \zeta_{H+/n} = dn_{H+} \cdot \nu_{H+}^{-1}$	Mol	Eq. 8n
e : Advancement e	$d_{tr} \zeta_{H+/e}$	$d_{tr} \zeta_{H+/e} = de_{H+} \cdot \nu_{H+}^{-1}$	C	Eq. 8e
Power	P_{tr}	$P_{tr} = I_{tr} \cdot F_{tr} = \partial_{tr} G \cdot \partial t$	$\text{W} = \text{J} \cdot \text{s}^{-1}$	Eq. 9

Eq. 4e: Scalar potential difference across the mitochondrial membrane. In a scalar electric transformation (flux of charge or current from the matrix space to the intermembrane and extramitochondrial space) the motive force is the difference of charge. The endergonic direction of translocation is defined in **Fig. 2** as $\text{H}^+_{\text{in}} \rightarrow \text{H}^+_{\text{out}}$.

Eq. 4n: $F=96.5 \text{ (kJ} \cdot \text{mol}^{-1})/\text{V}$.

Eq. 5: Note that the electric partial force is independent of temperature (Eq. 4), but the chemical partial force depends on absolute temperature, T .

Eq. 5e: RT is the gas constant times absolute temperature. $\ln(10) \cdot RT/F = 59.16$ and 61.54 mV at 25 and 37 °C, respectively.

Eq. 5n: $\ln(10) \cdot RT = 5.708$ and $5.938 \text{ kJ} \cdot \text{mol}^{-1}$ at 25 and 37 °C, respectively.

Eq. 6: $\partial_{tr} G$ [J] is the partial Gibbs *energy change* in the advancement of any transformation, tr.

Eq. 7: For $\text{X}=\text{C}$, flow is electric current, I_{el} [$\text{A} = \text{C} \cdot \text{s}^{-1}$].

Eq. 8: For a chemical reaction, the advancement of reaction r is $d_r\zeta_B = d_r n_B \cdot \nu_B^{-1}$ [mol]. The stoichiometric number is $\nu_B = -1$ or $\nu_B = 1$, depending on B being a product or substrate, respectively, in reaction r involving one mole of B. The conjugated *intensive* molar quantity, $F_{r,B} = \partial G / \partial r \zeta_B$ [$\text{J} \cdot \text{mol}^{-1}$], is the chemical force of reaction or *reaction-motive* force per stoichiometric amount of B. In reaction kinetics, $d_r n_B$ is expressed as a volume-specific quantity, which is the partial contribution to the total concentration change of B, $d_r c_B = d_r n_B / V$ and $dc_B = dn_B / V$, respectively. In open systems with constant volume V , $dc_B = d_r c_B + d_e c_B$, where r indicates the *internal* reaction and e indicates the *external* flux of B into the unit volume of the system. At steady state the concentration does not change, $dc_B = 0$, when $d_r c_B$ is compensated by the external flux of B, $d_r c_B = -d_e c_B$ (Gnaiger 1993b). Alternatively, $dc_B = 0$ when B is held constant by different coupled reactions in which B acts as a substrate or a product.

Eq. 6 to 9: B is the isomorphic motive unit or transformant in the transformation tr . $X = \text{mol}$ or C in proton translocation. For comparison, in a mechanical, vectorial advancement, $d_{me}\xi$ [m], the unit of the *force* is newton, F_{me} [$\text{N} = \text{J} \cdot \text{m}^{-1}$], and *flow* is the velocity, $v = d_{me}\xi / dt$ [$\text{m} \cdot \text{s}^{-1}$], such that the flow \cdot force product yields mechanical power, P_{me} [W] (Cohen *et al.* 2008). The corresponding *vectorial flux* (flow density per area) is velocity per cross-sectional area [$\text{s}^{-1} \cdot \text{m}^{-1}$]. The *scalar flux* lacks spatial information in a given volume, such that flux ($\text{m} \cdot \text{s}^{-1}$ per volume [$\text{s}^{-1} \cdot \text{m}^{-2}$]) times force yields volume-specific power, P_{Vme} [$\text{W} \cdot \text{m}^{-3}$].

3.3. Forces and flows in physics and irreversible thermodynamics

According to definition in physics, a potential difference and as such the *protonmotive force*, Δp_{H^+} , is not a force (Cohen *et al.* 2008). The fundamental forces of physics are distinguished from *motive forces* of statistical and irreversible thermodynamics.

Complementary to the attempt towards unification of fundamental forces defined in physics, the concepts of Nobel laureates Lars Onsager, Erwin Schrödinger, Ilya Prigogine and Peter Mitchell (even if expressed in apparently unrelated terms) unite the diversity of *generalized* or ‘isomorphic’ *flow-force* relationships, the product of which links to the dissipation function and Second Law of thermodynamics (Schrödinger 1944; Prigogine 1967). A *motive force* is the change of potentially available or ‘free’ energy (exergy) per isomorphic *motive* unit (force=exergy/motive unit; in integral form, this definition takes care of non-isothermal processes). In the framework of flow-force relationships, a potential difference is an isomorphic force, F_{tr} , involved in an exergy transformation, defined as the *partial* derivative of Gibbs energy per advancement of the transformation (**Table 5**, Eq. 6). This formal generalization represents an appreciation of the conceptual beauty of Peter Mitchell’s innovation of the protonmotive force against the background of the established paradigm of the electromotive force (emf) defined at the limit of zero current (Cohen *et al.* 2008). Perhaps the first account of a *motive force* in energy transformation can be traced back to the Peripatetic school around 300 BC in the context of moving a lever, up to Newton’s motive force proportional to the alteration of motion (Coopersmith 2010).

Vectorial and scalar forces, and fluxes: In chemical reactions and osmotic or diffusion processes occurring in a closed heterogeneous system, such as a chamber containing isolated mitochondria, scalar transformations occur without measured spatial direction but between separate compartments (translocation between the matrix and intermembrane space) or between energetically separated chemical substances (reactions from substrates to products). Hence the corresponding fluxes are not vectorial but scalar, and are expressed per volume and not per membrane area. The corresponding motive forces are also scalar potential *differences* across the membrane (**Table 5**), without taking into account the *gradients* across the 6 nm thick inner mitochondrial membrane (Rich 2003).

Coupling, efficiency, and power: In energetics (ergodynamics) coupling is defined as an exergy transformation fuelled by an exergonic (downhill) input process driving the advancement of an endergonic (uphill) output process. The (negative) output/input power ratio is the efficiency of a coupled energy transformation. Power is closely linked to the dissipation function (Prigogine 1967) and is the product of flow times isomorphic force (**Table 5**, Eq. 9; Gnaiger 1993b). At the limit of maximum efficiency of a completely coupled system, the (negative) input power equals the (positive) output power, such that the total power equals zero at an efficiency of 1.

Coupled versus bound processes: Since the chemiosmotic theory explains the mechanism of coupling in OXPHOS, it may be interesting to ask if the electric and chemical parts of proton translocation are coupled processes. This is not the case according to the definition of coupling. If the coupling mechanism is disengaged, the output process becomes independent of the input process, and both proceed in their downhill (exergonic) direction (**Fig. 2**). It is not possible to physically uncouple the electric and chemical processes, which are only *theoretically* partitioned as electric and chemical components and can be measured separately. If partial processes are non-separable, *i.e.* cannot be uncoupled, then these are not *coupled* but are defined as *bound* processes. The electric and chemical part are tightly bound partial forces of the protonmotive force, since the flow cannot be partitioned but only expressed in either electric or chemical isomorphic format (**Table 4**).

3.4. Normalization: flows and fluxes

Application of common and generally defined units is required for direct transfer of reported results into a database. The second [s] is the *SI* unit for the base quantity *time*. It is also the standard time-unit used in solution chemical kinetics. **Table 6** lists some conversion factors to obtain *SI* units. The term *rate* is too general and not useful for a database (**Fig. 7**). The inconsistency of the meanings of rate becomes fully apparent when considering Galileo

Galilei's famous principle, that 'bodies of different weight all fall at the same rate (have a constant acceleration)' (Coopersmith 2010).

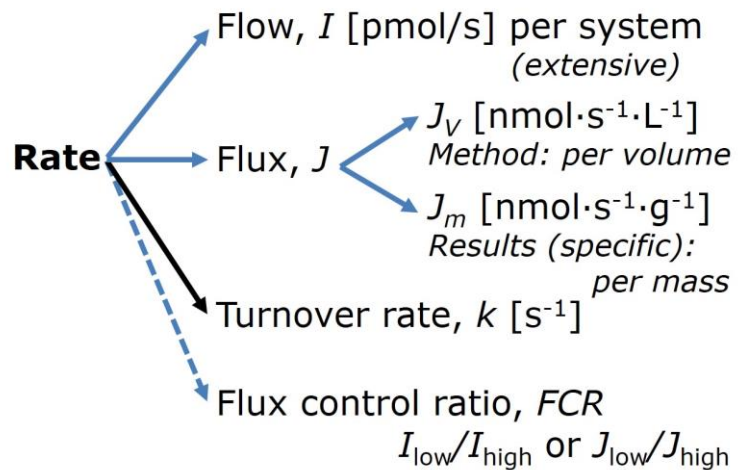
Extensive quantities: An extensive quantity increases proportionally with system size. The magnitude of an extensive quantity is completely additive for non-interacting subsystems, such as mass or flow expressed per defined system. The magnitude of these quantities depends on the extent or size of the system (Cohen *et al.* 2008).

Size-specific quantities: 'The adjective *specific* before the name of an extensive quantity is often used to mean *divided by mass*' (Cohen *et al.* 2008). A mass-specific quantity (*e.g.* mass-specific flux is flow divided by mass of the system) is independent of the extent of non-interacting homogenous subsystems. Tissue-specific quantities are of fundamental interest in comparative mitochondrial physiology, where *specific* refers to the *type* rather than *mass* of the tissue. The term *specific*, therefore, must be clarified further, such that tissue mass-specific (*e.g.* muscle mass-specific) quantities are defined.

Molar quantities: 'The adjective *molar* before the name of an extensive quantity generally means *divided by amount of substance*' (Cohen *et al.* 2008). The notion that all molar quantities then become *intensive* causes ambiguity in the meaning of *molar Gibbs energy*. It is important to emphasize the fundamental difference between normalization for *amount of substance* B in a system, where concentration is $c_B = n_B/V$ and the *rate of concentration change* is dc_B/dt [$\text{mol}\cdot\text{L}^{-1}\cdot\text{s}^{-1}$], *vs.* normalization for *amount of motive substance*, where the volume-specific flux of chemical reaction r is $d_r\zeta_B/dt\cdot V^{-1}$ [$(\text{mol}\cdot\text{s}^{-1})\cdot\text{L}^{-1}$]. These fundamentally different quantities have the same units, yet it is helpful to make the subtle distinction between [$\text{mol}\cdot\text{L}^{-1}\cdot\text{s}^{-1}$] and [$\text{mol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}$]. When the Gibbs energy of a system, G [J], is divided by the amount of substance B in the system, n_B [mol], a *size-specific* molar quantity is obtained, $G_B = G/n_B$ [$\text{J}\cdot\text{mol}^{-1}$], which is not any force at all. In contrast, when the partial Gibbs energy change, $\partial_r G$ [J], is divided by the motive amount of substance B in reaction r (advancement of reaction), $\partial_r\zeta_B$ [mol], the resulting *intensive* molar quantity, $F_{r,B} =$

$\partial G/\partial \xi_B$ [$\text{J}\cdot\text{mol}^{-1}$], is the chemical force of reaction r involving 1 mol B (**Table 5**, Note to Eq. 8).

Fig. 7. Different meanings of rate may lead to confusion, if the normalization is not sufficiently specified. Results are frequently expressed as mass-specific *flux*, J_m , per mg protein, dry or wet weight



(mass). Cell volume, V_{cell} , or mitochondrial volume, V_{mt} , may be used for normalization (volume-specific flux, $J_{V_{\text{cell}}}$ or $J_{V_{\text{mt}}}$), which then must be clearly distinguished from flux, J_V , expressed for methodological reasons per volume of the measurement system.

Flow per system, I : In analogy to electric terms, flow as an extensive quantity (I ; per system) is distinguished from flux as a size-specific quantity (J ; per system size) (**Fig. 7**). Electric current is flow, I_{el} [$\text{A} = \text{C}\cdot\text{s}^{-1}$] per system (extensive quantity). When dividing this extensive quantity by system size (membrane area), a size-specific quantity is obtained, which is electric flux (electric current density), J_{el} [$\text{A}\cdot\text{m}^{-2} = \text{C}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$].

Size-specific flux, J : Metabolic O_2 flow per muscle increases as muscle mass is increased. Muscle mass-specific O_2 flux should be independent of the size of the tissue sample studied in the instrumental chamber, but volume-specific O_2 flux (per volume of the instrumental chamber, V) should increase in direct proportion to the amount of sample in the chamber. Accurate definition of the experimental system is decisive: the experimental chamber is the closed, open, isothermal or non-isothermal *system* with defined volume as part of the measurement apparatus, in contrast to the experimental *sample* in the chamber (**Table**

6). Volume-specific O₂ flux depends on mass-concentration of the sample in the chamber, but should be independent of the chamber volume.

Flux per chamber volume, J_V : In open systems, external flows (such as O₂ supply) are distinguished from internal transformations (metabolic flow, O₂ consumption). In a closed system, external flows of all substances are zero and O₂ consumption (internal flow), I_{O_2} [pmol·s⁻¹], causes a decline of the amount of O₂ in the system, n_{O_2} [nmol]. Normalization of these quantities for the volume of the system, V [L=dm³], yields volume-specific O₂ flux, $J_{V,O_2}=I_{O_2}/V$ [nmol·s⁻¹·L⁻¹], and O₂ concentration, $[O_2]$ or $c_{O_2} = n_{O_2}/V$ [nmol·mL⁻¹=μmol·L⁻¹=μM]. Instrumental background O₂ flux is due to external flux into a non-ideal closed respirometer, such that total volume-specific flux has to be corrected for instrumental background O₂ flux. J_{V,O_2} is relevant mainly for methodological reasons and should be compared with the accuracy of instrumental resolution of background-corrected flux, *e.g.* ±1 nmol·s⁻¹·L⁻¹ (Gnaiger 2001). ‘Metabolic’ indicates background corrected O₂ flux.

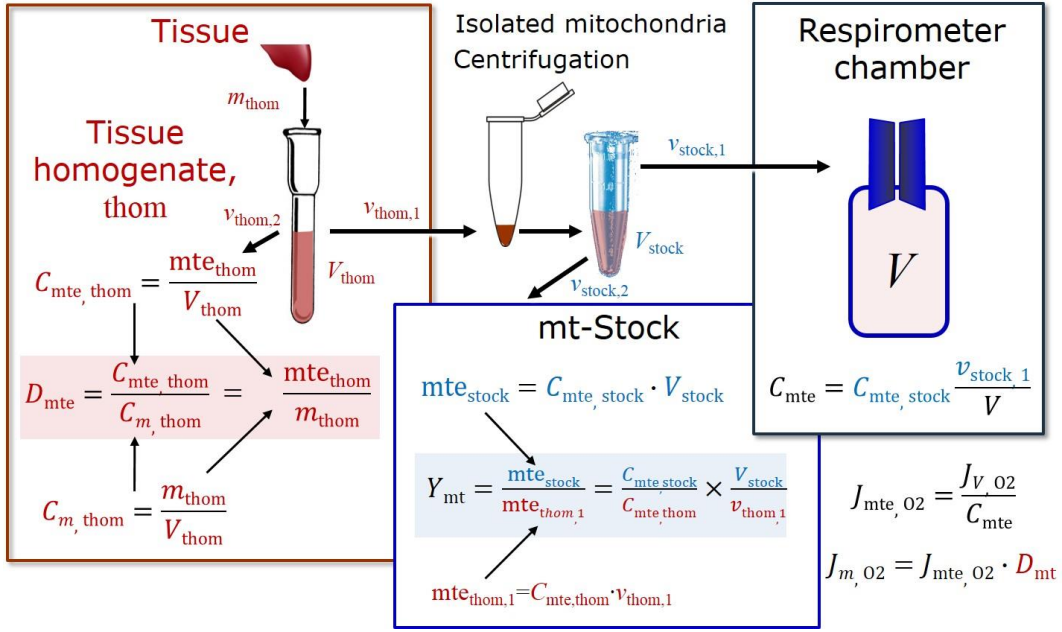


Fig. 8. Normalization of volume specific flux of isolated mitochondria and tissue homogenate.

A: Mitochondrial yield, Y_{mt} , in preparation of isolated mitochondria. Specific mt-density, D_{mte} ; quantity of mt-marker, mte ; mt-concentration, C_{mte} . $v_{thom,1}$ and $v_{stock,1}$ are the volumes transferred from the total volume, V_{thom} and V_{stock} , respectively. $mte_{thom,1}$ is the

amount of mitochondrial elements in volume $v_{thom,1}$ used for isolation. **B:** In respirometry with homogenate, $v_{thom,1}$ is transferred directly into the respirometer chamber. See **Table 6** for explanation of symbols.

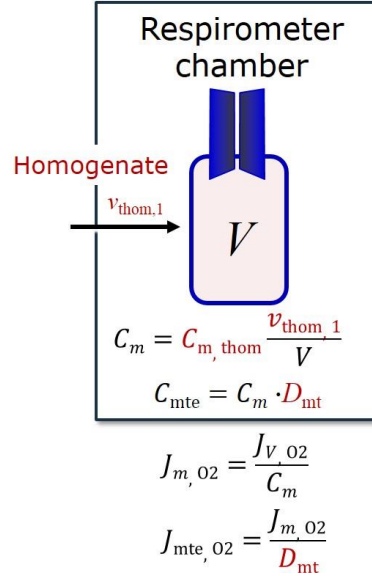


Table 6. Sample concentrations and normalization of flux.

Expression	Symbol	Definition	Unit	Notes
Sample				
Entity of sample	X	Cells, animals, patients		
Number of sample entities X	N_X	Number of cells, <i>etc.</i>	X	
Mass of sample X	m_X		g	
Mass of entity X	M_X	$M_X = m_X \cdot N_X^{-1}$	$g \cdot X^{-1}$	
Mitochondria				
Mitochondria	mt	$X=mt$		
Amount of mt-elements	mte	Quantity of mt-marker	x_{mte}	
Concentrations				
Sample number concentration	C_{NX}	$C_{NX} = N_X \cdot V^{-1}$	$X \cdot L^{-1}$	1
Sample mass concentration	C_{mX}	$C_{mX} = m_X \cdot V^{-1}$	$g \cdot L^{-1}$	
Mitochondrial concentration	C_{mte}	$C_{mte} = mte \cdot V^{-1}$	$x_{mte} \cdot L^{-1}$	2
Specific mitochondrial density	D_{mte}	$D_{mte} = mte \cdot m_X^{-1}$	$x_{mte} \cdot g^{-1}$	3
Mitochondrial content, mte per entity X	mte_X	$mte_X = mte \cdot N_X^{-1}$	$x_{mte} \cdot X^{-1}$	4
O₂ flow and flux				
Flow	I_{O_2}	Internal flow	$nmol \cdot s^{-1}$	5
Volume-specific flux	J_{V,O_2}	$J_{V,O_2} = I_{O_2} \cdot V^{-1}$	$nmol \cdot s^{-1} \cdot L^{-1}$	6
Flow per sample entity X	I_{X,O_2}	$I_{X,O_2} = J_{V,O_2} \cdot C_{NX}^{-1}$	$nmol \cdot s^{-1} \cdot X^{-1}$	7
Mass-specific flux	J_{mX,O_2}	$J_{mX,O_2} = J_{V,O_2} \cdot C_{mX}^{-1}$	$nmol \cdot s^{-1} \cdot g^{-1}$	8
Mitochondria-specific flux	J_{mte,O_2}	$J_{mte,O_2} = J_{V,O_2} \cdot C_{mte}^{-1}$	$nmol \cdot s^{-1} \cdot x_{mte}^{-1}$	9

1 In case $X=cells$, $C_{Ncell} = N_{cell} \cdot V^{-1}$ [$cell \cdot L^{-1}$].

2 mt-concentration is an experimental variable, dependent on sample concentration: (1) $C_{mte} = mte \cdot V^{-1}$; (2) $C_{mte} = mte_X \cdot C_{NX}$; (3) $C_{mte} = C_{mX} \cdot D_{mte}$.

3 If the amount of mitochondria, mte , is expressed as mitochondrial mass, then D_{mte} is the mass fraction of mitochondria in the sample. If mte is expressed as mitochondrial volume, V_{mt} , and the mass of sample, m_X , is replaced by volume of sample, V_X , then D_{mte} is the volume fraction of mitochondria in the sample.

4 $mte_X = mte \cdot N_X^{-1} = C_{mte} \cdot C_{NX}^{-1}$.

5 Entity O_2 can be replaced by other chemical entities B to study different reactions.

6 I_{O_2} and V are defined per instrumental chamber as a system of constant volume (and constant temperature), which may be closed or open. I_{O_2} is abbreviated for I_{t,O_2} , *i.e.* the

metabolic or internal O_2 flow of the chemical reaction r in which O_2 is consumed, hence the negative stoichiometric number, $\nu_{O_2} = -1$. $I_{r,O_2} = d_r n_{O_2} / dt \cdot \nu_{O_2}^{-1}$. If r includes all chemical reactions in which O_2 participates, then $d_r n_{O_2} = dn_{O_2} - d_e n_{O_2}$, where dn_{O_2} is the change of the amount of O_2 in the instrumental chamber and $d_e n_{O_2}$ is the amount of O_2 added externally to the system. At steady state, by definition $dn_{O_2} = 0$, hence $d_r n_{O_2} = -d_e n_{O_2}$.

- 7 J_{V,O_2} is an experimental variable, expressed per volume of the instrumental chamber.
- 8 I_{X,O_2} is a physiological variable, depending on the size of entity X .
- 9 There are many ways to normalize for a mitochondrial marker, which are used in different experimental approaches: (1) $J_{mte,O_2} = J_{V,O_2} \cdot C_{mte}^{-1}$; (2) $J_{mte,O_2} = J_{V,O_2} \cdot C_{mX}^{-1} \cdot D_{mte}^{-1} = J_{mX,O_2} \cdot D_{mte}^{-1}$; (3) $J_{mte,O_2} = J_{V,O_2} \cdot C_{NX}^{-1} \cdot mte_X^{-1} = I_{X,O_2} \cdot mte_X^{-1}$; (4) $J_{mte,O_2} = I_{O_2} \cdot mte^{-1}$.

Sample concentration C_{mX} : Normalization for sample concentration is required for reporting respiratory results. Consider a tissue or cells as the sample, X , and the sample mass, m_X [g] from which a mitochondrial preparation is obtained. The sample mass is frequently measured as wet or dry weight ($m_X \equiv W_w$ or W_d [g]), or as amount of tissue or cell protein ($m_X \equiv m_{Protein}$). In the case of permeabilized tissues, cells and homogenate, the sample concentration, $C_{mX} = m_X / V$ [$g \cdot L^{-1}$], is simply the mass of the subsample of tissue that is transferred into the instrumental chamber. Part of the mitochondria from the tissue are lost during preparation of isolated mitochondria, and only a fraction of mitochondria is obtained, expressed as the mitochondrial yield (**Fig. 8**). At a high mitochondrial yield the sample of isolated mitochondria is more representative of the total mitochondrial population than when the yield is low. Determination of the mitochondrial yield is based on measurement of the concentration of a mitochondrial marker in the tissue homogenate, $C_{mte,thom}$, which provides simultaneously information on the specific mitochondrial density in the sample (**Fig. 8**).

Mass-specific flux, J_{mX,O_2} : Mass-specific flux is obtained by normalization of respiration per mass of sample, m_X [g]. X is the type of sample, *e.g.* tissue homogenate,

permeabilized fibres or cells. Divide volume-specific flux by mass concentration of X , $J_{mX,O_2} = J_{V,O_2}/C_{mX}$, or flow per cell by mass per cell, $J_{mcell,O_2} = I_{cell,O_2}/M_{cell}$. If mass-specific O_2 flux is constant and independent of sample size (expressed as mass), then there is no interaction between the subsystems. A 1.5 mg and a 3.0 mg muscle sample respire at identical mass-specific flux. Mass-specific O_2 flux, however, may change with the mass of a tissue sample, cells or isolated mitochondria in the measuring chamber, in which case the nature of the interaction becomes an issue.

Mitochondrial concentration, C_{mte} , and mitochondrial markers: *‘Mitochondria are the structural and functional elemental units of cell respiration’* (Gnaiger 2014). Quantification of the amount of mitochondria depends on measurement of mitochondrial markers. The amount of a mitochondrial marker can be considered as the measurement of the amount of *elemental mitochondrial units* or *mitochondrial elements*, mte . However, since mitochondrial quality changes under certain stimuli and particularly in mitochondrial dysfunction, some markers can vary while other markers are unchanged. (1) Structural markers are mitochondrial volume or membrane area. Mitochondrial protein mass is a marker frequently used for isolated mitochondria. (2) Mitochondrial marker enzymes (amounts or activities) and molecular markers can be selected as matrix markers (*e.g.* citrate synthase activity, mtDNA) or inner mt-membrane markers (*e.g.* cytochrome *c* oxidase activity, aa_3 content; TOM20). (3) Extending the measurement of mitochondrial marker enzyme activity to mitochondrial pathway capacity, ETS or OXPHOS capacity can be considered as an integrative functional mitochondrial marker.

Depending on the type of mitochondrial marker, the mitochondrial elements, mte , are expressed in marker-specific units. Although concentration and density are used synonymously in physical chemistry, it is recommended to distinguish *experimental mitochondrial concentration*, $C_{mte}=mte/V$ and *physiological mitochondrial density*, $D_{mte}=mte/m_X$. Then mitochondrial density is the amount of mitochondrial elements per mass of

tissue. The former is mitochondrial density multiplied by sample mass concentration, $C_{\text{mte}}=D_{\text{mte}}\cdot C_{mX}$, or mitochondrial content multiplied by sample number concentration, $C_{\text{mte}}=\text{mte}_X\cdot C_{NX}$ (**Table 6**).

Mitochondria-specific flux, $J_{\text{mte},\text{O}_2}$: Volume-specific metabolic O_2 flux depends on: (1) the sample concentration in the volume of the instrumental chamber, C_{mX} , or C_{NX} ; (2) the mitochondrial density in the sample, $D_{\text{mte}}=\text{mte}/m_X$ or $\text{mte}_X=\text{mte}/N_X$; and (3) the specific mitochondrial activity or performance per elemental mitochondrial unit, $J_{\text{mte},\text{O}_2}=J_{V,\text{O}_2}/C_{\text{mte}}$ (**Table 6**). Obviously, the numerical results for $J_{\text{mte},\text{O}_2}$ vary depending on the type of mitochondrial marker chosen for measurement of mte and $C_{\text{mte}}=\text{mte}/V$. Some problems are common for all mitochondrial markers: (1) Accuracy of measurement is crucial, since even a highly accurate and reproducible measurement of O_2 flux becomes biased and noisy if normalized for a biased and noisy measurement of a mitochondrial marker. This problem is avoided when O_2 fluxes measured in substrate-uncoupler-inhibitor titration protocols are normalized for flux in a defined respiratory reference state, which is used as an *internal* marker and yields flux control ratios, *FCR* (**Fig. 7**). *FCR* are independent of any *externally* measured markers and are, therefore, statistically very robust. *FCR* indicate qualitative changes of mitochondrial respiratory control, with highest quantitative resolution, separating the effect of mitochondrial density or concentration on J_{mX,O_2} or I_{X,O_2} from that of function per elemental mitochondrial marker, $J_{\text{mte},\text{O}_2}$ (Pesta *et al.* 2011; Gnaiger 2014). (2) If mitochondrial quality does not change and only mitochondrial density varies as a determinant of mass-specific flux, then any marker is equally qualified and selection of the optimum marker depends only on the accuracy of measurement of the mitochondrial marker. (3) If mitochondrial quality changes, then there may not be any best mitochondrial marker.

Normalization is a problematic subject and it is crucial to consider the question of the study. If the study aims to compare tissue performance, such as the effects of a certain treatment on a specific muscle, then normalization can successfully be done to *e.g.* tissue

mass or protein content. If the aim, however, is to find differences of mitochondrial function independent of mitochondrial density (**Table 6**), then normalization to a mitochondrial marker is imperative. It is crucial to first establish that the marker chosen is not selectively altered by the performed treatment. In conclusion, the normalization must reflect the question at hand to allow a satisfying answer.

Number concentration, C_{NX} : The experimental *number concentration* of sample in the case of cells or animals (*e.g.* nematodes) is $C_{NX}=N_X/V$ [$X \cdot L^{-1}$], where N_X is the number of cells or animals in the chamber (**Table 6**).

Flow per sample entity, I_{X,O_2} : A special case of normalization is encountered in respiratory studies with permeabilized (or intact) cells. If respiration is expressed per cell, the O_2 flow per measurement system is replaced by the O_2 flow per cell, I_{cell,O_2} (**Table 6**). O_2 flow can be calculated from volume-specific O_2 flux, J_{V,O_2} [$nmol \cdot s^{-1} \cdot L^{-1}$] (per V of the measurement chamber [L]), divided by the number concentration of cells, $C_{N_{ce}}=N_{ce}/V$ [$cell \cdot L^{-1}$], where N_{ce} is the number of cells in the chamber. Cellular O_2 flow can be compared between cells of identical size. To take into account changes and differences in cell size, further normalization is required to obtain cell size-specific or mitochondrial marker-specific O_2 flux (Renner *et al.* 2003).

Many different units have been used to report the rate of oxygen consumption, OCR (**Tables 7 and 8**). For cellular studies we recommend that O_2 flow be expressed in units of attomole (10^{-18} mol) of O_2 consumed by each cell in a second [$amol \cdot s^{-1} \cdot cell^{-1}$], numerically equivalent to [$pmol \cdot s^{-1} \cdot 10^{-6}$ cells]. This convention allows information to be easily used when designing experiments in which oxygen uptake must be considered. For example, to estimate the volume-specific O_2 flux in a instrumental chamber that would be expected at a particular cell number concentration, one simply needs to multiply the flow per cell by the number of cells per volume of interest. This provides the amount of O_2 [mol] consumed per time [s^{-1}] per unit volume [L^{-1}]. At an O_2 flow of $100 amol \cdot s^{-1} \cdot cell^{-1}$ and a cell density of $10^9 cells \cdot L^{-1}$ (10^6

cells·mL⁻¹), the volume-specific O₂ flux is 100 nmol·s⁻¹·L⁻¹ (100 pmol·s⁻¹·mL⁻¹). Because the litre is the basic unit of volume for concentration and is used for most solution chemical kinetics, if one multiplies $I_{\text{cell},\text{O}_2}$ by $C_{N_{\text{cell}}}$, then the result will not only be the amount of O₂ [mol] consumed per time [s⁻¹] in one litre [L⁻¹], but also the change in the concentration of oxygen per second (for any volume of an ideally closed system). This is ideal for kinetic modeling as it blends with chemical rate equations where concentrations are typically expressed in mol·L⁻¹ (Wagner *et al.* 2011).

The complexity changes when the sample is a whole organism studied as experimental model. The well-established scaling law in respiratory physiology reveals a strong interaction of O₂ consumption and individual body mass of an organism, since *basal* metabolic rate (flow) does not increase linearly with body mass, whereas *maximum* mass-specific O₂ flux, $\dot{V}_{\text{O}_2\text{max}}$, or $\dot{V}_{\text{O}_2\text{peak}}$, is approximately constant across a large range of individual body mass (Weibel and Hoppeler 2005), with individuals, breeds, and certain species deviating substantially from this general relationship. $\dot{V}_{\text{O}_2\text{peak}}$ of human endurance athletes is 60 to 80 mL O₂·min⁻¹·kg⁻¹ body mass, converted to $J_{m,\text{O}_2\text{peak}}$ of 45 to 60 nmol·s⁻¹·g⁻¹ (**Table 7**).

3.5. Conversion: oxygen, protons, ATP

J_{O_2} is coupled in mitochondrial steady states to proton cycling, $J_{\infty\text{H}^+} = J_{\text{H}^+\text{out}} = J_{\text{H}^+\text{in}}$ (**Fig. 2**). $J_{\text{H}^+\text{out}/n}$ and $J_{\text{H}^+\text{in}/n}$ [nmol·s⁻¹·L⁻¹] are converted into electric units, $J_{\text{H}^+\text{out}/e}$ [mC·s⁻¹·L⁻¹=mA·L⁻¹] = $J_{\text{H}^+\text{out}/n}$ [nmol·s⁻¹·L⁻¹]· F [C·mol⁻¹]·10⁻⁶ (**Table 4**). At a $J_{\text{H}^+\text{out}}/J_{\text{O}_2}$ ratio or $\text{H}^+_{\text{out}}/\text{O}_2$ of 20 ($\text{H}^+_{\text{out}}/\text{O}=10$), a volume-specific O₂ flux of 100 nmol·s⁻¹·L⁻¹ would correspond to a proton flux of 2,000 nmol H⁺_{out}·s⁻¹·L⁻¹ or volume-specific current of 193 mA·L⁻¹.

$$J_{V,\text{H}^+\text{out}/e} [\text{mA}\cdot\text{L}^{-1}] = J_{V,\text{H}^+\text{out}/n} \cdot F \cdot 10^{-6} [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}\cdot\text{mC}\cdot\text{nmol}^{-1}] \quad (\text{Eq. 10.1})$$

$$J_{V,\text{H}^+\text{out}/e} [\text{mA}\cdot\text{L}^{-1}] = J_{V,\text{O}_2} \cdot (\text{H}^+_{\text{out}}/\text{O}_2) \cdot F \cdot 10^{-6} [\text{mC}\cdot\text{s}^{-1}\cdot\text{L}^{-1}=\text{mA}\cdot\text{L}^{-1}] \quad (\text{Eq. 10.2})$$

ETS capacity in various human cell types including HEK 293, primary HUVEC and fibroblasts ranges from 50 to 180 amol·s⁻¹·cell⁻¹ (see Gnaiger 2014). At 100 amol·s⁻¹·cell⁻¹

corrected for ROX (corresponding to a catabolic power of $-48 \text{ pW}\cdot\text{cell}^{-1}$), the current across the mt-membranes, I_e , approximates $193 \text{ pA}\cdot\text{cell}^{-1}$ or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of -110 W .

Table 7. Conversion of various units used in respirometry and ergometry. e is the number of electrons or reducing equivalents. z_B is the charge number of entity B.

1 Unit	x	Multiplication factor	SI-Unit
ng.atom $\text{O}\cdot\text{s}^{-1}$	(2 e)	0.5	nmol $\text{O}_2\cdot\text{s}^{-1}$
ng.atom $\text{O}\cdot\text{min}^{-1}$	(2 e)	8.33	pmol $\text{O}_2\cdot\text{s}^{-1}$
natom $\text{O}\cdot\text{min}^{-1}$	(2 e)	8.33	pmol $\text{O}_2\cdot\text{s}^{-1}$
nmol $\text{O}_2\cdot\text{min}^{-1}$	(4 e)	16.67	pmol $\text{O}_2\cdot\text{s}^{-1}$
nmol $\text{O}_2\cdot\text{h}^{-1}$	(4 e)	0.2778	pmol $\text{O}_2\cdot\text{s}^{-1}$
ml $\text{O}_2\cdot\text{min}^{-1}$ at STPD		0.744	$\mu\text{mol O}_2\cdot\text{s}^{-1}$
W = J/s at -470 kJ/mol O_2		-2.128	$\mu\text{mol O}_2\cdot\text{s}^{-1}$
mA = $\text{mC}\cdot\text{s}^{-1}$	($z_B=1$)	10.36	nmol $\text{B}\cdot\text{s}^{-1}$
nmol $\text{B}\cdot\text{s}^{-1}$	($z_B=1$)	0.09649	mA

Table 8. Conversion for units with preservation of numerical values. For prefixes see Table 9.

Name	Frequently used unit	Equivalent unit
Volume-specific flux, J_{V,O_2}	$\text{pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$ $\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-3}$	$\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}$ $\text{mmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}$
Cell-specific flow, I_{O_2}	$\text{pmol}\cdot\text{s}^{-1}\cdot 10^{-6} \text{ cells}$	$\text{amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$
Cell density, C_{ce}	$10^6 \text{ cells}\cdot\text{mL}^{-1}$	$10^9 \text{ cells}\cdot\text{L}^{-1}$
Mass-specific flux, J_{m,O_2}	$\text{pmol}\cdot\text{s}^{-1}\cdot\text{mg}^{-1}$	$\text{nmol}\cdot\text{s}^{-1}\cdot\text{g}^{-1}$
Catabolic power, P_{k,O_2}	$\mu\text{W}\cdot 10^{-6} \text{ cells}$	$\text{pW}\cdot\text{cell}^{-1}$
Volume	L mL	dm^3 cm^3
Amount of substance concentration	$\text{M} = \text{mol}\cdot\text{L}^{-1}$	$\text{mol}\cdot\text{dm}^{-3}$

Table 9. SI prefixes (IUPAC).

Submultiple	Prefix	Symbol	Multiple	Prefix	Symbol
10^{-3}	milli	m	10^3	kilo	k
10^{-6}	micro	μ	10^6	mega	M
10^{-9}	nano	n	10^9	giga	G
10^{-12}	pico	p	10^{12}	tera	T
10^{-15}	femto	f	10^{15}	peta	P
10^{-18}	atto	a	10^{18}	exa	E
10^{-21}	zepto	z	10^{21}	zetta	Z

For NADH- and succinate-linked respiration, the mechanistic »P/O₂ ratio (referring to the full 4 electron reduction of O₂) is calculated at 20/3.7 and 12/3.7 (Eq. 11) equal to 5.4 and 3.3. The classical »P/O ratios (referring to the 2 electron reduction of 0.5 O₂) are 2.7 and 1.6 (Watt *et al.* 2010), in direct agreement with the measured »P/O ratio for succinate of 1.58 ± 0.02 (Gnaiger *et al.* 2000; for detailed reviews see Wikström and Hummer 2012; Sazanov 2015),

$$\text{»P/O}_2 = (\text{H}^+_{\text{out}}/\text{O}_2)/(\text{H}^+_{\text{in}}/\text{»P}) \quad (11)$$

In summary (**Fig. 1**),

$$J_{V,\text{»P}} [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}] = J_{V,\text{O}_2} \cdot (\text{H}^+_{\text{out}}/\text{O}_2) / (\text{H}^+_{\text{in}}/\text{»P}) \quad (12.1)$$

$$J_{V,\text{»P}} [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}] = J_{V,\text{O}_2} \cdot (\text{»P/O}_2) \quad (12.2)$$

Considering isolated mitochondria as powerhouses and proton pumps as molecular machines and relating the experimental results to energy metabolism of the intact cell, the cellular »P/O₂ based on oxidation of glycogen is increased by the glycolytic substrate-level phosphorylation of 3 »P/Glyc, *i.e.* 0.5 mol »P for each mol O₂ consumed in the complete oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial »P/O₂ ratio of 5.4 yields a bioenergetic cell physiological »P/O₂ ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, the energetic cost of which must potentially be taken into account. Considering also substrate-level phosphorylation in the TCA cycle, this high »P/O₂ ratio not only reflects proton

translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger 1993b).

4. Conclusions

MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory defects linked to genetic variations, age-related health risks, gender-specific mitochondrial performance, life style with its consequences on degenerative diseases, and environmental exposure to toxicological agents. The present recommendations on coupling control (Part 1) will be extended in a series of manuscripts on pathway control of mitochondrial respiration, respiratory states in intact cells, and harmonization of experimental procedures.

The optimal choice for expressing O₂ flow per biological system, and normalization for specific tissue-markers (volume, mass, protein) and mitochondrial markers (volume, protein, content, mtDNA, activity of marker enzymes, respiratory reference state) is guided by the scientific question. Interpretation of the data obtained depends critically on the appropriate normalization, and reporting rates merely as nmol·s⁻¹ is discouraged because it is uninformative. For studies with intact or permeabilized cells, we recommend that normalizations be provided as far as possible: (1) on a per cell basis as O₂ flow (a biophysical normalization); (2) per mg cell protein or per cell mass as mass-specific O₂ flux (a cellular normalization); and (3) per mitochondrial marker as mt-specific flux (a mitochondrial normalization). With information on cell size and the use of both normalizations the maximal potential information is available (Renner *et al.* 2003; Wagner *et al.* 2011; Gnaiger 2014). When using isolated mitochondria, mitochondrial protein is a frequently applied mitochondrial marker, the use of which is basically restricted to isolated mitochondria. Mitochondrial markers must be evaluated critically, such as citrate synthase activity as an enzymatic matrix marker, which provide a link to the tissue of origin on the basis of

calculating the mitochondrial yield, *i.e.* the fraction of mitochondrial marker obtained from a unit mass of tissue.

To provide an overall perspective of mitochondrial physiology we may link cellular bioenergetics to systemic human respiratory activity, without yet addressing cell- and tissue-specific mitochondrial function. A routine O₂ flow of 234 μmol·s⁻¹ per individual or flux of 3.3 nmol·s⁻¹·g⁻¹ body mass corresponds to -110 W catabolic energy flow at a body mass of 70 kg and -470 kJ/mol O₂. Considering a cell count of 514·10⁶ cells per g tissue mass (Ahluwalia 2017), the average O₂ flow per cell at $J_{m,O_2\text{peak}}$ of 45 nmol·s⁻¹·g⁻¹ (60 mL O₂·min⁻¹·kg⁻¹) is 88 amol·s⁻¹·cell⁻¹, which compares well with OXPHOS capacity of human fibroblasts (not ETS but the lower OXPHOS capacity is used as a reference; Gnaiger 2014). We can describe our body as the sum of 37·10¹² cells (37 trillion cells; Bianconi 2013). An estimate of mitochondrial content at 300 mitochondria per cell (West *et al.* 2002) raises questions on the concept of mitochondrial number (**Table 8**). Mitochondrial fitness of our 11·10¹⁵ mitochondria (11 quadrillion mt) is indicated if O₂ flow of 0.02 amol·s⁻¹·mt⁻¹ at rest can be activated to 0.3 amol·s⁻¹·mt⁻¹ at high ergometric performance.

Acknowledgements

Financially supported by Supported by COST Action CA15203 MitoEAGLE and K-Regio project MitoFit (EG).

Competing financial interests: E.G. is founder and CEO of Oroboros Instruments, Innsbruck, Austria.

References (*incomplete; www links will be deleted in the final version*)

Ahluwalia A. Allometric scaling in-vitro. Sci Rep 2017;7:42113.

Altmann R. Die Elementarorganismen und ihre Beziehungen zu den Zellen. Zweite vermehrte Auflage. Verlag Von Veit & Comp, Leipzig 1894;160 pp. -

www.mitoeagle.org/index.php/Altmann_1894_Verlag_Von_Veit_%26_Comp

Bianconi E, Piovesan A, Facchin F, Beraudi A, Casadei R, Frabetti F, Vitale L, Pelleri MC, Tassani S, Piva F, Perez-Amodio S, Strippoli P, Canaider S. (2013) An estimation of the number of cells in the human body. Ann Hum Biol. 40(6):463-471. -
<http://dx.doi.org/10.3109/03014460.2013.807878> PMID: 23829164

Brown GC. Control of respiration and ATP synthesis in mammalian mitochondria and cells. Biochem J 1992;284:1-13. - www.mitoeagle.org/index.php/Brown_1992_Biochem_J

Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation: III. The steady state. J Biol Chem 1955;217:409-27. -
www.mitoeagle.org/index.php/Chance_1955_J_Biol_Chem-III

Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. IV. The respiratory chain. J Biol Chem 1955;217:429-38. -
www.mitoeagle.org/index.php/Chance_1955_J_Biol_Chem-IV

Chance B, Williams GR. The respiratory chain and oxidative phosphorylation. Adv Enzymol Relat Subj Biochem 1956;17:65-134. -
www.mitoeagle.org/index.php/Chance_1956_Adv_Enzymol_Relat_Subj_Biochem

Cohen ER, Cvitas T, Frey JG, Holmström B, Kuchitsu K, Marquardt R, Mills I, Pavese F, Quack M, Stohner J, Strauss HL, Takami M, Thor HL. Quantities, Units and Symbols in Physical Chemistry, IUPAC Green Book 2008;3rd Edition, 2nd Printing, IUPAC & RSC Publishing, Cambridge. -
www.mitoeagle.org/index.php/Cohen_2008_IUPAC_Green_Book

Coopersmith J. Energy, the subtle concept. The discovery of Feynman's blocks from Leibnitz to Einstein. Oxford University Press 2010;400 pp.

Dufour S, Rousse N, Canioni P, Diolez P. Top-down control analysis of temperature effect on oxidative phosphorylation. *Biochem J* 1996;314:743-51.

Ernster L, Schatz G Mitochondria: a historical review. *J Cell Biol* 1981;91:227s-55s. -

www.mitoeagle.org/index.php/Ernster_1981_J_Cell_Biol

Estabrook RW. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods Enzymol* 1967;10:41-7. -

www.mitoeagle.org/index.php/Estabrook_1967_Methods_Enzymol

Fell D. Understanding the control of metabolism. Portland Press 1997.

Garlid KD, Semrad C, Zinchenko V. Does redox slip contribute significantly to mitochondrial respiration? In: Schuster S, Rigoulet M, Ouhabi R, Mazat J-P (eds) Modern trends in biothermokinetics. Plenum Press, New York, London 1993;287-93.

Gnaiger E. Efficiency and power strategies under hypoxia. Is low efficiency at high glycolytic ATP production a paradox? In: Surviving Hypoxia: Mechanisms of Control and Adaptation. Hochachka PW, Lutz PL, Sick T, Rosenthal M, Van den Thillart G (eds.) CRC Press, Boca Raton, Ann Arbor, London, Tokyo 1993a:77-109. -

www.mitoeagle.org/index.php/Gnaiger_1993_Hypoxia

Gnaiger E. Nonequilibrium thermodynamics of energy transformations. *Pure Appl Chem* 1993b;65:1983-2002. - www.mitoeagle.org/index.php/Gnaiger_1993_Pure_Appl_Chem

Gnaiger E. Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respir Physiol* 2001;128:277-97. -

www.mitoeagle.org/index.php/Gnaiger_2001_Respir_Physiol

Gnaiger E. Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis. 4th ed. *Mitochondr Physiol Network* 2014;19.12. Oroboros MiPNet

Publications, Innsbruck:80 pp. -

www.mitoeagle.org/index.php/Gnaiger_2014_MitoPathways

Gnaiger E. Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. *Int J Biochem Cell Biol* 2009;41:1837-45. -

www.mitoeagle.org/index.php/Gnaiger_2009_Int_J_Biochem_Cell_Biol

Gnaiger E, Méndez G, Hand SC. High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. *Proc Natl Acad Sci USA* 2000;97:11080-5. -

www.mitoeagle.org/index.php/Gnaiger_2000_Proc_Natl_Acad_Sci_U_S_A

Hofstadter DR. Gödel, Escher, Bach: An eternal golden braid. A metaphorical fugue on minds and machines in the spirit of Lewis Carroll. Harvester Press 1979;499 pp. -

www.mitoeagle.org/index.php/Hofstadter_1979_Harvester_Press

Komlódi T, Tretter L. Methylene blue stimulates substrate-level phosphorylation catalysed by succinyl-CoA ligase in the citric acid cycle. *Neuropharmacology* 2017;123:287-98. -

www.mitoeagle.org/index.php/Komlodi_2017_Neuropharmacology

Lemieux H, Blier PU, Gnaiger E. Remodeling pathway control of mitochondrial respiratory capacity by temperature in mouse heart: electron flow through the Q-junction in permeabilized fibers. *Sci Rep* 2017;7:2840. -

www.mitoeagle.org/index.php/Lemieux_2017_Sci_Rep

Miller GA. The science of words. Scientific American Library New York 1991;276 pp. -

www.mitoeagle.org/index.php/Miller_1991_Scientific_American_Library

Mitchell P, Moyle J. Respiration-driven proton translocation in rat liver mitochondria. *Biochem J* 1967;105:1147-62. -

www.mitoeagle.org/index.php/Mitchell_1967_Biochem_J

Morrow RM, Picard M, Derbeneva O, Leipzig J, McManus MJ, Gousspillou G, Barbat-Artigas S, Dos Santos C, Hepple RT, Murdock DG, Wallace DC. Mitochondrial energy

deficiency leads to hyperproliferation of skeletal muscle mitochondria and enhanced insulin sensitivity. Proc Natl Acad Sci U S A 2017;114:2705-10. -

www.mitoeagle.org/index.php/Morrow_2017_Proc_Natl_Acad_Sci_U_S_A

Nicholls DG, Ferguson S. Bioenergetics 4. Elsevier 2013.

Prigogine I. Introduction to thermodynamics of irreversible processes. Interscience, New York, 1967;3rd ed.

Puchowicz MA, Varnes ME, Cohen BH, Friedman NR, Kerr DS, Hoppel CL. Oxidative phosphorylation analysis: assessing the integrated functional activity of human skeletal muscle mitochondria – case studies. Mitochondrion 2004;4:377-85. -

www.mitoeagle.org/index.php/Puchowicz_2004_Mitochondrion

Renner K, Amberger A, Konwalinka G, Gnaiger E. Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. Biochim Biophys Acta 2003;1642:115-23. -

www.mitoeagle.org/index.php/Renner_2003_Biochim_Biophys_Acta

Rich P. Chemiosmotic coupling: The cost of living. Nature 2003;421:583. -

www.mitoeagle.org/index.php/Rich_2003_Nature

Rostovtseva TK, Sheldon KL, Hassanzadeh E, Monge C, Saks V, Bezrukov SM, Sackett DL. Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. Proc Natl Acad Sci USA 2008;105:18746-51. -

www.mitoeagle.org/index.php/Rostovtseva_2008_Proc_Natl_Acad_Sci_U_S_A

Rustin P, Parfait B, Chretien D, Bourgeron T, Djouadi F, Bastin J, Rötig A, Munnich A. Fluxes of nicotinamide adenine dinucleotides through mitochondrial membranes in human cultured cells. J Biol Chem 1996;271:14785-90.

Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS. Permeabilised cell and skinned fiber techniques in studies

of mitochondrial function in vivo. *Mol Cell Biochem* 1998;184:81-100. -

http://www.mitoeagle.org/index.php/Saks_1998_Mol_Cell_Biochem

Sazanov LA. A giant molecular proton pump: structure and mechanism of respiratory

complex I. *Nat Rev Mol Cell Biol* 2015;16:375-88. -

www.mitoeagle.org/index.php/Sazanov_2015_Nat_Rev_Mol_Cell_Biol

Schrödinger E. What is life? The physical aspect of the living cell. Cambridge Univ Press,

1944. - www.mitoeagle.org/index.php/Gnaiger_1994_BTK

Stucki JW, Ineichen EA. Energy dissipation by calcium recycling and the efficiency of

calcium transport in rat-liver mitochondria. *Eur J Biochem* 1974;48:365-75.

Wagner BA, Venkataraman S, Buettner GR. The rate of oxygen utilization by cells. *Free*

Radic Biol Med. 2011;51:700-712.

<http://dx.doi.org/10.1016/j.freeradbiomed.2011.05.024> PMID: PMC3147247

Watt IN, Montgomery MG, Runswick MJ, Leslie AG, Walker JE. Bioenergetic cost of

making an adenosine triphosphate molecule in animal mitochondria. *Proc Natl Acad Sci*

U S A 2010;107:16823-7. -

www.mitoeagle.org/index.php/Watt_2010_Proc_Natl_Acad_Sci_U_S_A

Weibel ER, Hoppeler H. Exercise-induced maximal metabolic rate scales with muscle aerobic

capacity. *J Exp Biol* 2005;208:1635–44.

West GB, Woodruff WH, Brown JH. Allometric scaling of metabolic rate from molecules and

mitochondria to cells and mammals. *Proc Natl Acad Sci USA* 2002;99 Suppl 1:2473–8.

Wikström M, Hummer G. Stoichiometry of proton translocation by respiratory complex I and

its mechanistic implications. *Proc Natl Acad Sci U S A* 2012;109:4431-6. -

www.mitoeagle.org/index.php/Wikstroem_2012_Proc_Natl_Acad_Sci_U_S_A