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**Mitochondrial respiratory control and the protonmotive force:
a conceptual perspective on coupling states in mitochondrial preparations.**

MitoEAGLE recommendations Part 1

MitoEAGLE Terminology Group

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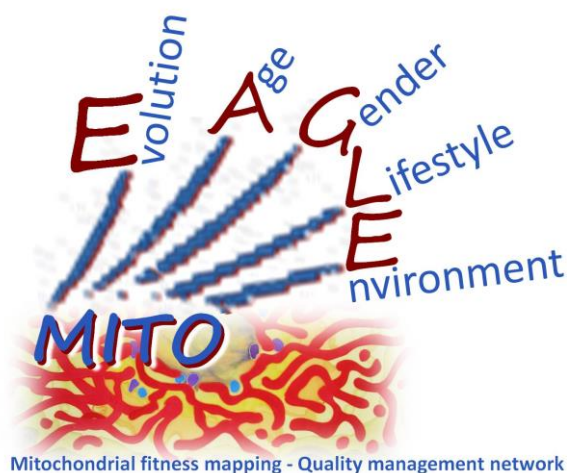
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*This manuscript on 'Mitochondrial respiratory control' is in preparation as a position statement in the frame of COST Action CA15203 MitoEAGLE. Up to now, the list of co-authors is mainly based on previous MitoEAGLE Working Group Meetings. In the **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.*



*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

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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 1 to 5 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*, OXPHOS, provides diagnostic reference values and is, therefore, measured at kinetically saturating concentrations of ADP and inorganic phosphate. 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 OXPHOS 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.

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.

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- * 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 small organelles of eukaryotic cells with a double membrane separating the intermembrane space and the matrix with tubular or disk-shaped cristae. 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 the reduction of O₂ is electrochemically coupled to conservation of energy in the form of ATP. Besides many other components present in mitochondria, 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, and fatty acid oxidation. Mitochondria maintain their nucleus-independent mtDNA. There is a constant crosstalk between these partially independent organelles and coordinated functions of the cell. Most of their proteins are encoded by nuclear DNA, and different cellular signaling pathways, such as Ca²⁺ and protein kinases, modulate mitochondrial activity and structure. In addition, mitochondria interact with each other by fusion and fission all affecting their activity and cell respiration. 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 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. 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 exerted in a mitochondrial preparation by 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 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).

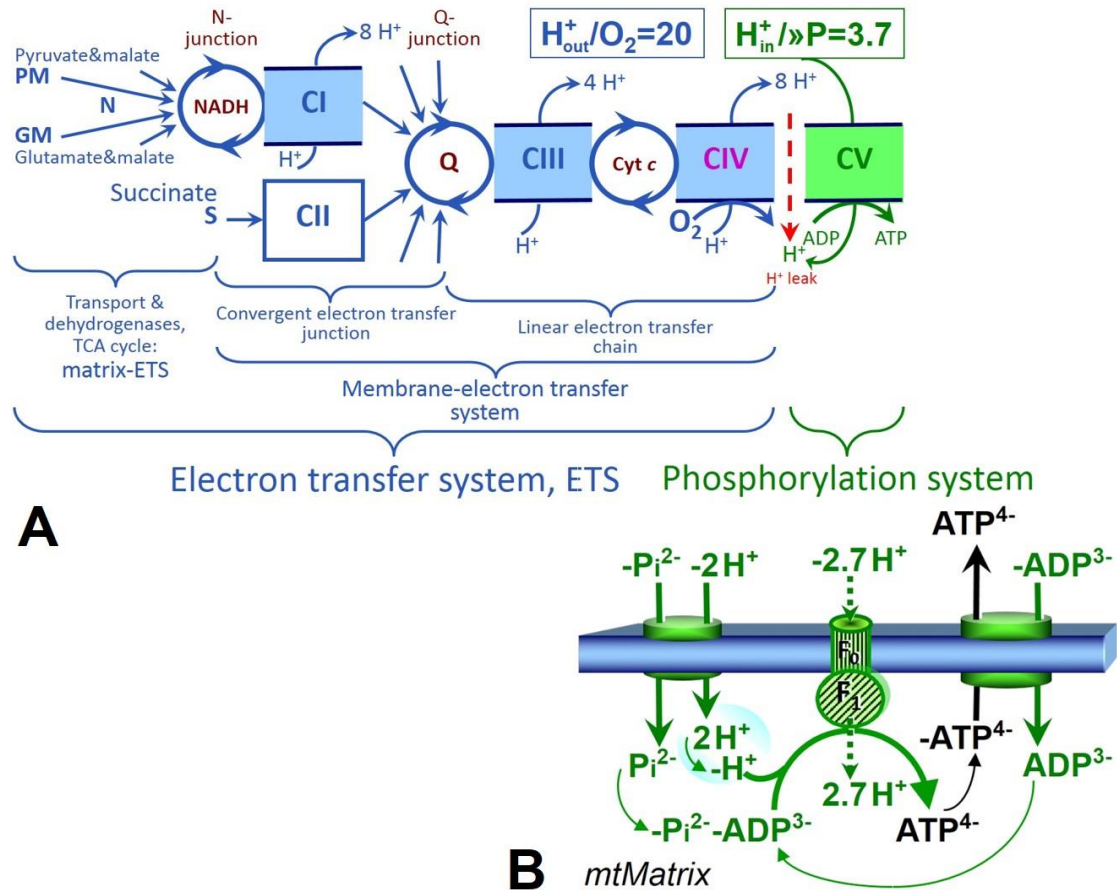


Fig. 1. The mitochondrial respiratory system. In oxidative phosphorylation, the electron transfer system, ETS (A; *to be updated by L Szalov*) is coupled to the phosphorylation system (B). See Eqs. 4 and 5 for further explanation. Modified from (A) Lemieux *et al.* (2017) and (B) Gnaiger (2014).

Phosphorylation, »P: *Phosphorylation* in the context of OXPHOS is clearly defined as phosphorylation of ADP to ATP. On the other hand, the term phosphorylation is used in the general literature in many different contexts (phosphorylation of enzymes, *etc.*). 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 »P for the energetic uphill direction of phosphorylation coupled to catabolic reactions, and likewise the symbol «P for the corresponding downhill reaction (**Fig. 2**). ATP synthase is the most important enzyme complex of the phosphorylation system (**Fig. 1B**), and »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, $2 \text{ ADP} \leftrightarrow \text{ATP} + \text{AMP}$, proceeds without fuel substrates in the presence of ADP (Kömlödi 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 (**Fig. 2**); (2) fuel substrate, pathway competition and oxygen availability (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, NADH/NAD⁺, coenzyme Q, cytochrome *c*); and (8) mitochondrial density (enzyme concentrations and membrane area) and morphology (fission and fusion). Evolutionary background genetic or acquired mutations causing mitochondrial dysfunction and gene

therapy; Age; Gender and hormone concentrations; Life style including exercise and nutrition; and Environmental 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).

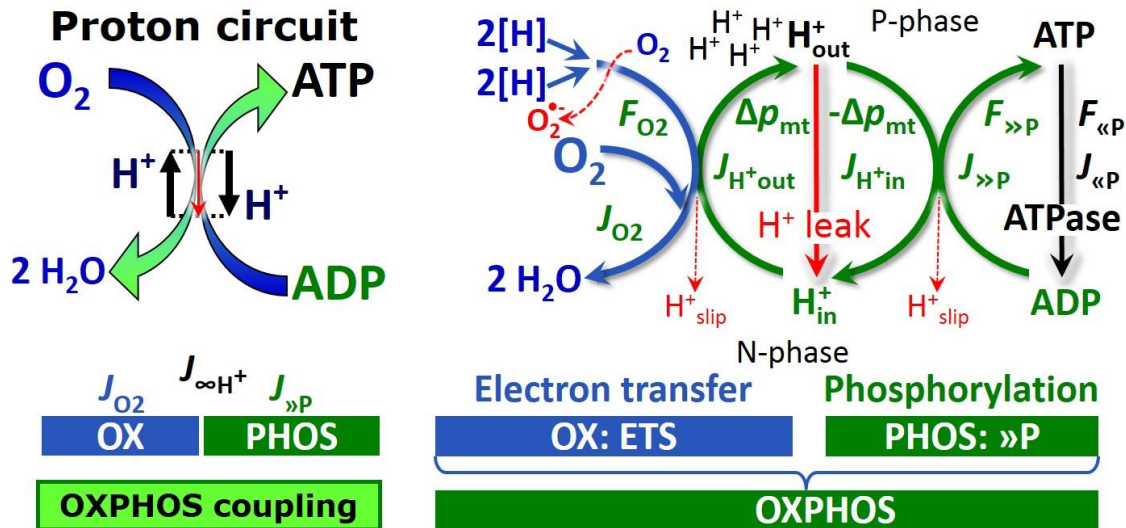


Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). Oxygen flux, J_{O_2} , is coupled to the phosphorylation of ADP to ATP , $J_{\gg P}$, by the proton pumps of the electron transfer system, ETS, pushing the outward proton flux, J_{H^+out} , and generating the protonmotive force, Δp_{mt} . ATP synthase is driven by the protonmotive force, $-\Delta p_{mt}$, 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 Δp_{mt} , 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 to superoxide anion radical ($O_2^{\cdot-}$). Modified from Gnaiger (2014).

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 μ M), 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 (confusion caused by earlier edition of the ‘Bioenergetics’ textbook by Nicholls and Ferguson, add reference?).

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.

State 4 is reached only if the mitochondrial preparation is of high quality and is 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 Δp_{mt} and high ATP/ADP ratio are maintained. State 4 respiration reflects intrinsic proton leak and the ATPase activity.

State 5 is a state obtained after exhaustion of oxygen in a closed respirometric chamber. Oxygen diffusion 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. 3 to 5**).

Table 2. Coupling states and ROX in mitochondrial preparations.

State	Respiration	Δp_{mt}	Induced by:	Limited by:
LEAK	<i>L</i> ; low, proton leak-dependent respiration	Max	Kinetically saturating [substrate] and [O ₂] without ADP or with full inhibition of the ADP phosphorylation system	Proton leak
OXPHOS	<i>P</i> ; high, ADP-stimulated respiration	High	Kinetically saturating [substrate], [ADP], [P _i] and [O ₂]	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	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 saturating concentrations of O_2 , respiratory and phosphorylation substrates, and zero uncoupler, to provide an estimate of the maximal capacity of OXPHOS in any given pathway control state. Respiratory capacities at

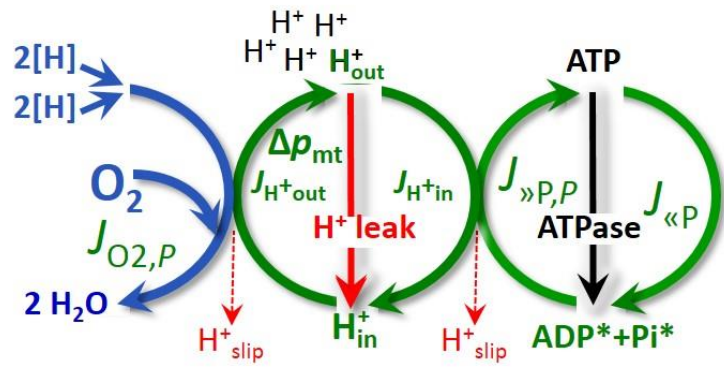


Fig. 3. OXPHOS state when phosphorylation, $J_{\gg P}$, is supported by a high Δp_{mt} , is stimulated by kinetically saturating $[ADP]^*$ and inorganic phosphate, $[Pi]^*$. O_2 flux, $J_{O_2,P}$, is highly coupled at a maximum $\gg P/O_2$ ratio, $J_{\gg P,P}/J_{O_2,P}$ (see also Fig. 2).

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 saturating concentrations of O_2 , respiratory substrate and optimum *exogenous* uncoupler concentration for maximum O_2 flux, as an estimate of oxidative ETS capacity. As a

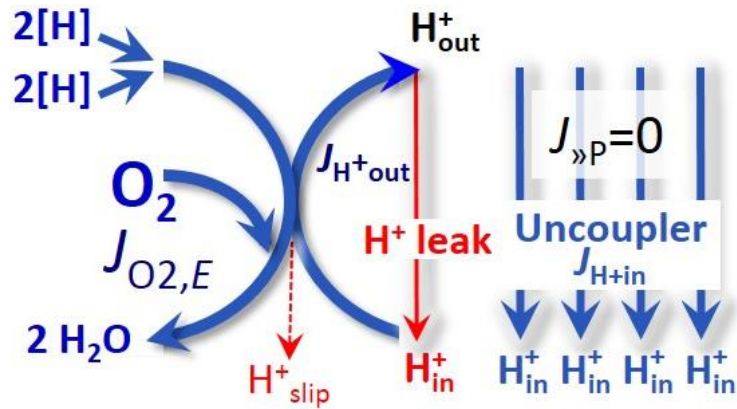


Fig. 4. ETS state when noncoupled respiration, $J_{O_2,E}$, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, $J_{»P}=0$ (see also Fig. 2).

consequence of the nearly collapsed Δp_{mt} , the driving force for phosphorylation is missing and $J_{»P}=0$. 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

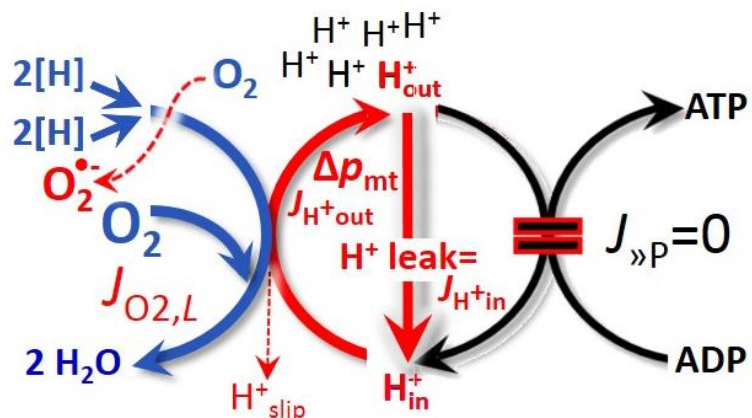


Fig. 5. LEAK state when phosphorylation is arrested, $J_{»P}=0$, and oxygen flux, $J_{O_2,L}$, is controlled mainly by the proton leak, which equals J_{H^+in} , at maximum Δp_{mt} (see also Fig. 2).

obtain an indirect estimate 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, or adenylate nucleotide translocase, such as carboxyatractyloside. Oxygen consumption in State 4 is an overestimation of LEAK respiration if the ATPase activity maintains some stimulation of respiration by recycled ADP at $J_{\text{P}} > 0$. This can be tested by inhibition of the phosphorylation system using oligomycin, ensuring that $J_{\text{P}} = 0$.

Proton leak: Proton leak is the *uncoupled* process in which protons are translocated across the inner mitochondrial membrane in the direction of the downhill protonmotive force without coupling to phosphorylation. The proton leak flux depends on Δp_{mt} , is a property of the inner mitochondrial membrane, and is physiologically controlled, *e.g.* in brown adipose tissue by UCP1. Mitochondrial injuries may lead to *dyscoupling* as a pathological or toxicological cause of *uncoupled* respiration, distinguished from the experimentally induced *noncoupled* respiration in the ETS state.

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. 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.

Besides these three main coupling states, 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

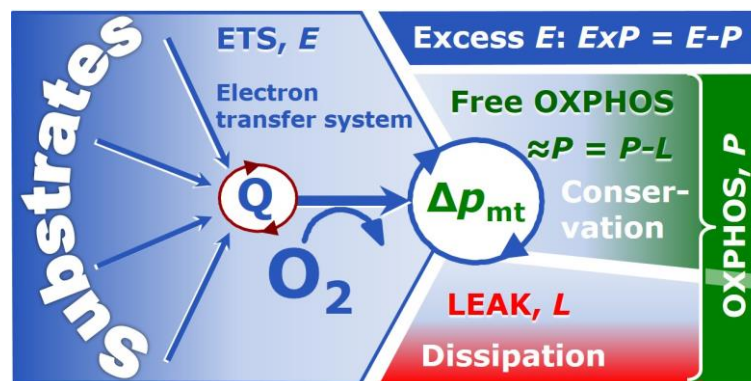
coupling states. ROX is not necessarily equivalent to non-mitochondrial respiration, considering oxygen-consuming reactions in mitochondria not related to ETS, such as oxygen consumption in the reaction catalyzed by monoamine oxidases. In the presence of O_2 , ROX is measured either in the absence of fuel substrates or after blocking the electron supply to cytochrome *c* oxidase and alternative oxidases.

3. States and rates

3.1. Respiratory states and respiratory rates

The protonmotive force is *high* in the OXPHOS state when it drives phosphorylation, *elevated* 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, OXPHOS, LEAK) and corresponding rates (E , P , L).



Modified from Gnaiger (2014).

Fig. 6 summarizes the three coupling states, ETS, LEAK and OXPHOS, 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 [Pi] (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_{mt} ; (2) the pathway control state with single or multiple electron input into the Q-junction and involvement of three or less coupling sites determining the $\text{H}^+_{\text{out}}/\text{O}_2$ *coupling stoichiometry* (**Fig. 1A**); and (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, Δp_{mt} , 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, which is *free* (corrected for LEAK respiration) and is fully coupled to phosphorylation with a maximum mechanistic stoichiometry, $\approx P = P - L$ (**Fig. 6**).

3.2. The steady-state and protonmotive force

Mitochondria represent thermodynamically open systems functioning as biochemical reaction systems in a non-equilibrium steady state. The non-equilibrium steady state maintains constant concentrations of metabolites at fluxes and chemical potential gradients different from zero (Qian 2006). Steady-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.* O₂ consumption) are instantaneously compensated by external flows (*e.g.* O₂ supply), such that oxygen concentration does not change in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored in closed systems may satisfy the criteria of pseudo-steady states for limited periods of time, when the changes occurring in the system (concentrations of O₂, fuel substrates, ADP) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require kinetically saturating concentrations of substrates to be maintained and thus depend on the kinetics of the processes under investigation.

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

$$\Delta p_{\text{mt}} = \Delta \Psi_{\text{mt}} + \Delta \mu_{\text{H}^+}/F \quad (1)$$

consists of an electric part, $\Delta \Psi_{\text{mt}}$, which is the difference of charge (electric potential difference), and of a chemical part, $\Delta \mu_{\text{H}^+}$, which stems from the difference of pH (chemical potential difference) and incorporates the Faraday constant (**Table 3**).

Faraday constant, F : The Faraday constant is the product of elementary charge ($e = 1.602177 \cdot 10^{-19} \cdot \text{C}$) and the Avogadro (Loschmidt) constant ($N_{\text{A}} = 6.022136 \times 10^{23} \cdot \text{mol}^{-1}$), $F = eN_{\text{A}} = 96,485.3 \text{ C/mol}$. The Faraday constant yields the conversion between protonmotive force, $F_e = \Delta p_{\text{mt}} [\text{J/C}]$, expressed per *motive charge*, $e [\text{C}]$, and protonmotive force or

electrochemical (chemiosmotic) potential difference, $F_n = \Delta\tilde{\mu}_{\text{H}^+} = \Delta p_{\text{mt}} \cdot F$ [J/mol], expressed per motive amount of protons, n [mol],

$$F_n = F_e \cdot F \quad (2)$$

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* (**Table 3**).

Table 3. Protonmotive force matrix. Rows: Force is the sum of partial isomorphic protonmotive forces (el and d), but conjugated flow cannot be partitioned. Columns: Electric or chemical isomorphic format (e or n). The Faraday constant, $F = eN_A = 96,485.3 \text{ C}\cdot\text{mol}^{-1}$, converts protonmotive force from isomorph e to n , $F_n = F_e \cdot eN_A$ (Eqs. 1 to 4). Flow x force = $I_{e,\text{H}^+} \cdot F_e = I_{n,\text{H}^+} \cdot F_n = \text{Power [J/s=W]}$. The signs depend on the definition of the compartmental direction of the translocation (Fig. 2). Proton charge, e , and amount of substance, n , define the units for isomorph e and n .

State	Force	=	electric	+	chemical	Unit	
Protonmotive force, e	Δp_{mt}	=	$\Delta\Psi_{\text{mt}}$	+	$\Delta\mu_{\text{H}^+}/F$	J/C	
Chemiosmotic potential, n	$\Delta\tilde{\mu}_{\text{H}^+}$	=	$\Delta\Psi_{\text{mt}} \cdot F$	+	$\Delta\mu_{\text{H}^+}$	J/mol	
State	Isomorph i	Force, F_i	=	el	+	d	
	Electric charge, e	F_e	=	$F_{e,\text{el}}$	+	$F_{e,\text{d}}$	J/C
	Amount of substance, n	F_n	=	$F_{n,\text{el}}$	+	$F_{n,\text{d}}$	J/mol
Rate	Isomorph i	Flow, I_i	=	e	or	n	
	Electric charge, e			I_{e,H^+}		C/s	
	Amount of substance, n				I_{n,H^+}	mol/s	

Electric part of the protonmotive force, el: $F_{e,\text{el}} = \Delta\Psi_{\text{mt}}$ is the electric part of the protonmotive force expressed in units joule per coulomb, *i.e.* volt [V=J/C], defined as partial Gibbs energy change per motive elementary charge of protons, e [C].

Chemical diffusion (translocation) part of the protonmotive force, d: $F_{n,d} = \Delta\mu_{H^+}$ 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].

Isomorph e [C]: $F_{e,d} = \Delta\mu_{H^+}/F$ is the chemical force expressed in units joule per coulomb [J/C=V], defined as partial Gibbs energy change per *motive amount of protons expressed in units of electric charge*, e [C],

$$F_e = F_{e,el} + F_{e,d} \quad [\text{J/C}] \quad (3)$$

Isomorph n [mol]: $F_{n,el} = \Delta\Psi_{mt} \cdot F$ is the electric force expressed in units joule per mole [J/mol], defined as partial Gibbs energy change per *motive amount of charge*, n [mol],

$$F_n = F_{n,el} + F_{n,d} \quad [\text{J/mol}] \quad (4)$$

Protonmotive means that protons are moved across the mitochondrial membrane at constant force. The direction of translocation is defined in **Fig. 2** as $H^+_{in} \rightarrow H^+_{out}$,

$$F_{n,d} = \Delta\mu_{H^+} = -\ln(10) \cdot RT \cdot \Delta p H_{mt} \quad (5)$$

where RT is the gas constant times absolute temperature. $\ln(10) \cdot RT = 5.708$ and 5.938 $\text{kJ} \cdot \text{mol}^{-1}$ at 25 and 37 °C, respectively. $\ln(10) \cdot RT/F = 59.16$ and 61.54 mV at 25 and 37 °C, respectively. For a ΔpH of 1 unit, the chemical force (Eq. 5) changes by 6 $\text{kJ} \cdot \text{mol}^{-1}$ and the protonmotive force (Eq. 3) changes by 0.06 V.

Since F equals 96.5 ($\text{kJ} \cdot \text{mol}^{-1}$)/V, a membrane potential difference of -0.2 V (Eq. 3) equals an electrochemical potential difference, $\Delta\tilde{\mu}_{H^+}$, of 19 $\text{kJ} \cdot \text{mol}^{-1}$ H^+_{out} (Eq. 4). Considering a driving force of -470 $\text{kJ} \cdot \text{mol}^{-1}$ O_2 for oxidation, the thermodynamic limit of the H^+_{out}/O_2 ratio is reached at a value of $470/19 = 24$, compared with a mechanistic stoichiometry of 20 ($H^+_{out}/O=10$).

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_{mt} , 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). A potential difference is, in the framework of flow-force relationships, an isomorphic force, F_{tr} , involved in an exergy transformation, defined as the *partial* derivative of Gibbs energy, $\partial_{\text{tr}}G$, per advancement, $\partial_{\text{tr}}\xi$, of the transformation, tr (the isomorphic motive unit in the transformation): $F_{\text{tr}} = \partial_{\text{tr}}G/\partial_{\text{tr}}\xi$ (Gnaiger 1993a,b). 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).

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, expressed in units [$\text{J}\cdot\text{mol}^{-1}$] as potential *differences* across the membrane, without taking into

account the *gradients* across the 6 nm thick inner mitochondrial membrane (Rich 2003). 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, $\Delta\Psi_{\text{mt}}$ [$\text{V}=\text{J}\cdot\text{C}^{-1}$]. For comparison, in a mechanical, vectorial advancement, $d_{\text{me}}\zeta$ [m], the unit of the *force* is newton, F_{me} [$\text{N}=\text{J}\cdot\text{m}^{-1}$], and *flow* is the velocity, $v = d_{\text{me}}\zeta/dt$ [$\text{m}\cdot\text{s}^{-1}$], such that the flow-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, $P_{V\text{me}}$ [$\text{W}\cdot\text{m}^{-3}$].

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, $P_{\text{tr}} = \partial_{\text{tr}}G/dt$ [$\text{W}=\text{J}\cdot\text{s}^{-1}$], is closely linked to the dissipation function (Prigogine 1967) and is the product of flow, $I_{\text{tr}}=d_{\text{tr}}\zeta\cdot dt^{-1}$ [$\text{x}_{\text{tr}}\cdot\text{s}^{-1}$] times isomorphic force, $F_{\text{tr}} = \partial_{\text{tr}}G/\partial_{\text{tr}}\zeta$ [$\text{J}\cdot\text{x}_{\text{tr}}^{-1}$] (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 isomorphous format (**Table 3**).

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 5** lists some conversion factors to obtain *SI* units. The term *rate* is too general and not useful for a database (**Fig. 7**).

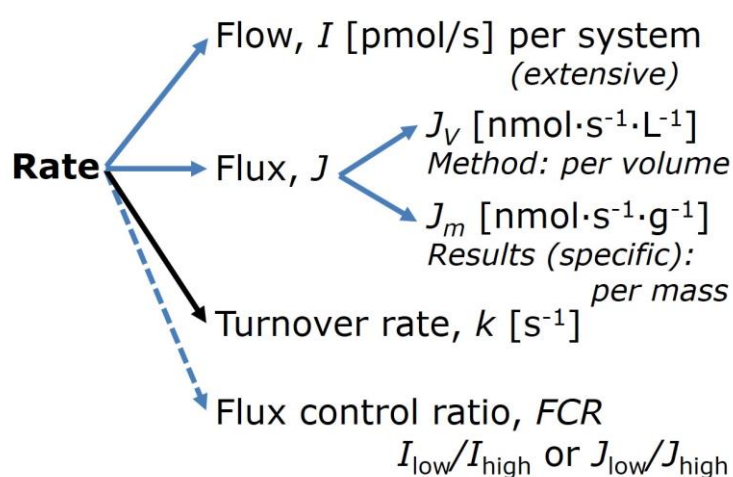
Extensive quantities: An extensive quantity increases proportionally with system size. The magnitude of an extensive quantity is completely additive for non-interacting subsystems, such as mass or flow expressed per defined system. The magnitude of these quantities depends on the extent or size of the 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 [(mol·L⁻¹)·s⁻¹], vs. normalization for *amount of motive substance*, where the volume-specific flux of chemical reaction *r* is $d_r \xi_B / dt \cdot V^{-1}$ [(mol·s⁻¹)·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), $\partial_r \xi_B = \nu_B \cdot \partial_r n_B$ [mol], the resulting *intensive* molar quantity, $F_{r,B} = \partial_r G / \partial_r \xi_B$ [$\text{J}\cdot\text{mol}^{-1}$], is the chemical force of reaction r involving 1 mol B (the stoichiometric number is $\nu_B = -1$ or $\nu_B = 1$, depending on B being a product or substrate, respectively, in reaction r).

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_{ce} , or mitochondrial volume, V_{mt} , may be used for normalization (volume-specific flux, J_{Vce} or J_{Vmt}), 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 (the cross-sectional area of a wire or area of a membrane), a

size-specific quantity is obtained, which is electric flux (electric current density), J_{el} [$A \cdot m^{-2} = C \cdot s^{-1} \cdot m^{-2}$].

Size-specific flux, J : 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. Volume-specific O_2 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 O_2 supply) are distinguished from internal transformations (metabolic flow, O_2 consumption). In a closed system, external flows of all substances are zero and O_2 consumption (internal flow), I_{O_2} [$pmol \cdot s^{-1}$], causes a decline of the amount of O_2 in the system, n_{O_2} [$nmol$]. Normalization of these quantities for the volume of the system, V [$L=dm^3$], yields volume-specific O_2 flux, $J_{V,O_2} = I_{O_2}/V$ [$nmol \cdot s^{-1} \cdot L^{-1}$], and O_2 concentration, $[O_2]$ or $c_{O_2} = n_{O_2}/V$ [$nmol \cdot mL^{-1} = \mu mol \cdot L^{-1} = \mu M$]. Instrumental background O_2 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 O_2 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. $\pm 1 nmol \cdot s^{-1} \cdot L^{-1}$ (Gnaiger 2001). ‘Metabolic’ indicates background corrected O_2 flux.

Table 4. 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	$X=mt$		
Elemental marker for amount of mt	em_{mt}	Quantity of mt -marker	x_{mt}	
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_{mt}	$C_{mt} = em_{mt} \cdot V^{-1}$	$x_{mt} \cdot L^{-1}$	2
Specific mitochondrial density	D_{mt}	$D_{mt} = em_{mt} \cdot m_X^{-1}$	$x_{mt} \cdot g^{-1}$	3
Mitochondrial content, em_{mt} of entity X	em_{mtX}	$em_{mtX} = em_{mt} \cdot N_X^{-1}$	$x_{mt} \cdot X^{-1}$	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_{mt,O_2}	$J_{mt,O_2} = J_{V,O_2} \cdot C_{mt}^{-1}$	$nmol \cdot s^{-1} \cdot x_{mt}^{-1}$	9

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

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

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

4 $em_{mtX} = C_{mt} \cdot C_{NX}^{-1}$.

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) $J_{mt,O_2} = J_{V,O_2} \cdot C_{mt}^{-1}$; (2) $J_{mt,O_2} = J_{V,O_2} \cdot C_{mX}^{-1} \cdot D_{mt}^{-1} = J_{mX,O_2} \cdot D_{mt}^{-1}$; (3) $J_{mt,O_2} = J_{V,O_2} \cdot C_{NX}^{-1} \cdot em_{mtX}^{-1} = I_{X,O_2} \cdot em_{mtX}^{-1}$; (4) $J_{mt,O_2} = I_{O_2} \cdot em_{mt}^{-1}$.

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. **Add isolated mitochondria and mitochondrial yield as a link to the tissue of origin.**

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, $J_{mX,O_2} = I_{cell,O_2} / M_{cell}$. 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.

Mitochondrial concentration, C_{mt} , 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 defines the amount of *elemental mitochondrial units*, em_{mt} : (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 elemental mitochondrial unit, em_{mt} , is expressed in marker-specific units $[x_{mt}]$. Although concentration and density are used synonymously in physical chemistry, it is recommended to distinguish *experimental mitochondrial concentration*, $C_{mt}=em_{mt}/V$ $[x_{mt}\cdot L^{-1}]$ and *physiological mitochondrial density*, D_{mt} . Then mitochondrial density is the amount of mitochondrial elemental marker per mass of tissue. The former is sample mass concentration multiplied by mitochondrial density, $C_{mt}=C_{mX}\cdot D_{mt}$, or sample number concentration multiplied by mitochondrial content, $C_{mt}=C_{NX}\cdot em_{mtX}$ (**Table 4**).

Mitochondria-specific flux, J_{mt,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, $D_{mt}=em_{mt}/m_X$ or $em_{mtX}=em_{mt}/N_X$; and (3) the specific mitochondrial activity or performance per elemental mitochondrial unit, $J_{mt,O_2}=J_{V,O_2}/C_{mt}$ (**Table 4**). Obviously, the numerical results for J_{mt,O_2} vary depending on the type of mitochondrial marker chosen for measurement of em_{mt} and $C_{mt}=em_{mt}/V$. Some problems are common for all mitochondrial markers: (1) Accuracy of measurement is crucial, since even a

highly accurate and reproducible measurement of O₂ flux becomes biased and noisy if normalized for a biased and noisy measurement of a mitochondrial marker. This problem is avoided when O₂ 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_{mt,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 to 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 4**), 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 4**).

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

O₂ flow per measurement system is replaced by the O₂ flow per cell, $I_{\text{cell},\text{O}_2}$ (**Table 4**). O₂ flow can be calculated from volume-specific O₂ flux, J_{V,O_2} [$\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}$] (per V of the measurement chamber [L]), divided by the number concentration of cells, $C_{N_{\text{ce}}}=N_{\text{ce}}/V$ [$\text{cell}\cdot\text{L}^{-1}$], where N_{ce} is the number of cells in the chamber. Cellular O₂ 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 O₂ flux (Renner et al. 2003).

Many different units have been used to report the rate of oxygen consumption, OCR (**Tables 5 and 6**). For cellular studies we recommend that O₂ flow be expressed in units of attomole (10^{-18} mol) of O₂ consumed by each cell in a second [$\text{amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$], numerically equivalent to [$\text{pmol}\cdot\text{s}^{-1}\cdot 10^{-6}$ 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 O₂ flux in a measurement 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 O₂ [mol] consumed per time [s^{-1}] per unit volume [L^{-1}]. At an O₂ flow of $100 \text{ amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$ and a cell density of $10^9 \text{ cells}\cdot\text{L}^{-1}$ ($10^6 \text{ cells}\cdot\text{mL}^{-1}$), the volume-specific O₂ flux is $100 \text{ nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}$ ($100 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$). 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 $\text{mol}\cdot\text{L}^{-1}$ (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, $\dot{V}_{O_2\max}$, or $\dot{V}_{O_2\text{peak}}$, 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. $\dot{V}_{O_2\text{peak}}$ of human endurance athletes is 60 to 80 mL O₂·min⁻¹·kg⁻¹ body mass, converted to $J_{m,O_2\text{peak}}$ of 45 to 60 nmol·s⁻¹·g⁻¹ (**Table 5**).

3.5. Conversion: oxygen, protons, ATP

J_{O_2} is coupled in mitochondrial steady states to proton cycling, $J_{\infty H^+} = J_{H^+\text{out}} = J_{H^+\text{in}}$ (**Fig. 2**). $J_{H^+\text{out}}$ and $J_{H^+\text{in}}$ [nmol·s⁻¹·L⁻¹] are converted into an electric flux (per volume), J_e [mC·s⁻¹·L⁻¹=mA·L⁻¹] = $J_{H^+\text{out}}$ [nmol·s⁻¹·L⁻¹]· F [C·mol⁻¹]·10⁻⁶ (**Table 3**). At a $J_{H^+\text{out}}/J_{O_2}$ ratio or $H^+\text{out}/O_2$ of 20 ($H^+\text{out}/O=10$), 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.

$$J_e [\text{mA}\cdot\text{L}^{-1}] = J_{H^+\text{out}}\cdot F\cdot 10^{-6} [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}\cdot\text{mC}\cdot\text{nmol}^{-1}] \quad (6.1)$$

$$J_e [\text{mA}\cdot\text{L}^{-1}] = J_{V,O_2}\cdot(H^+\text{out}/O_2)\cdot F\cdot 10^{-6} [\text{mC}\cdot\text{s}^{-1}\cdot\text{L}^{-1}=\text{mA}\cdot\text{L}^{-1}] \quad (6.2)$$

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 pW·cell⁻¹), the current across the mt-membranes, I_e , approximates 193 pA·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 5. 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 O \cdot s $^{-1}$	(2 e)	0.5	nmol O $_2$ \cdot s $^{-1}$
ng.atom O \cdot min $^{-1}$	(2 e)	8.33	pmol O $_2$ \cdot s $^{-1}$
natom O \cdot min $^{-1}$	(2 e)	8.33	pmol O $_2$ \cdot s $^{-1}$
nmol O $_2$ \cdot min $^{-1}$	(4 e)	16.67	pmol O $_2$ \cdot s $^{-1}$
nmol O $_2$ \cdot h $^{-1}$	(4 e)	0.2778	pmol O $_2$ \cdot s $^{-1}$
ml O $_2$ \cdot min $^{-1}$ at STPD		0.744	μ mol O $_2$ \cdot s $^{-1}$
W = J/s at -470 kJ/mol O $_2$		-2.128	μ mol O $_2$ \cdot s $^{-1}$
mA = mC \cdot s $^{-1}$	($z_B=1$)	10.36	nmol B \cdot s $^{-1}$
nmol B \cdot s $^{-1}$	($z_B=1$)	0.09649	mA

Table 6. Conversion for units with preservation of numerical values. For prefixes see Table 7.

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

Table 7. 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. 7) 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),

$$\text{»P/O}_2 = (\text{H}^+_{\text{out/O}_2})/(\text{H}^+_{\text{in/»P}}) \quad (7)$$

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

$$J_{V,\text{»P}} [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}] = J_{V,\text{O}_2} \cdot (\text{H}^+_{\text{out/O}_2}) / (\text{H}^+_{\text{in/»P}}) \quad (8.1)$$

$$J_{V,\text{»P}} [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}] = J_{V,\text{O}_2} \cdot (\text{»P/O}_2) \quad (8.2)$$

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 glycogen. 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 O_2 flow per biological system, and normalization for specific tissue-markers (volume, mass, protein) and mitochondrial markers (volume, protein, content, mtDNA, activity of marker enzymes) is guided by the scientific question. Interpretation of the data obtained depends critically on the appropriate normalization, and reporting rates merely as $\text{nmol}\cdot\text{s}^{-1}$ is discouraged. For studies with intact or permeabilized cells, we recommend that normalizations be provided as far as possible: (1) on a per cell basis as O_2 flow (a biophysical normalization); (2) per mg cell protein or per cell mass as mass-specific O_2 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 O_2 flow of $234 \mu\text{mol}\cdot\text{s}^{-1}$ per individual or flux of $3.3 \text{ nmol}\cdot\text{s}^{-1}\cdot\text{g}^{-1}$ body mass corresponds to -110 W catabolic energy flow at a body mass of 70 kg and $-470 \text{ kJ/mol } O_2$. Considering a cell count of $514\cdot 10^6$ cells per g tissue mass and an estimate of 300 mitochondria per cell (Ahluwalia 2017), the average O_2 flow per cell at $J_{m,O_2\text{peak}}$ of $45 \text{ nmol}\cdot\text{s}^{-1}\cdot\text{g}^{-1}$ ($60 \text{ ml } O_2\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$) is $88 \text{ amol}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$, 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 $36 \cdot 10^{12}$ cells (36 trillion cells). Mitochondrial fitness of our $11 \cdot 10^{15}$ mitochondria (11 quadrillion mt) is indicated if O_2 flow of $0.02 \text{ amol} \cdot \text{s}^{-1} \cdot \text{mt}^{-1}$ at rest can be activated to $0.3 \text{ amol} \cdot \text{s}^{-1} \cdot \text{mt}^{-1}$ at high ergometric performance.

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