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The protonmotive force and respiratory control:

Building blocks of mitochondrial physiology

Part 1.

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MitoEAGLE Network

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This manuscript on 'The protonmotive force and respiratory control' is a position statement in the frame of COST Action CA15203 MitoEAGLE. The list of co-authors evolved from MitoEAGLE Working Group Meetings and a ***bottom-up*** *spirit of COST in phase 1: 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 consensus statement on reporting data of mitochondrial respiration in terms of metabolic flows and fluxes. We plan a series of follow-up reports by the expanding MitoEAGLE Network, to increase the scope of recommendations on harmonization and facilitate global communication and collaboration.*

Phase 2 - until ***October 12:*** *We continue to invite comments and suggestions on the MitoEAGLE preprint, 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.*

To join as a co-author, please feel free to focus on a particular section in terms of direct input and references, contributing to the scope of the manuscript from the perspective of your expertise. Your comments will be largely posted on the discussion page of the MitoEAGLE preprint website.

If you prefer to submit comments in the format of a referee's evaluation rather than a contribution as a co-author, I will be glad to distribute your views to the updated list of co-authors for a balanced response. We would ask for your consent on this open bottom-up policy.

*We organize a MitoEAGLE session linked to our series of reports at the MiP*conference *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,

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Contents

Abstract

Clarity of concepts and consistency of nomenclature are trademarks of a research field across its specializations, facilitating transdisciplinary communication and education. As research and knowledge of mitochondrial physiology expand, the necessity for harmonizing nomenclature concerning mitochondrial respiratory states and rates has become apparent. Peter Mitchell's concept of the protonmotive force establishes the links between electrical and chemical components of energy transformation and coupling in oxidative phosphorylation. This unifying concept provides the framework for developing a consistent terminology of 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. Standards for evaluation of respiratory states must be followed for the development of databases of mitochondrial respiratory function in species, tissues and cells studied under diverse physiological and experimental conditions.

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

Box 1:

In brief: mitochondria and Bioblasts

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

Mitochondria are dynamic organelles contained within eukaryotic cells, with a double membrane. The inner mitochondrial membrane shows dynamic tubular and disk-shaped cristae that separate the mitochondrial matrix, *i.e.* the internal mitochondrial compartment, and the intermembrane space; the latter being enclosed by the outer mitochondrial membrane. Mitochondria were described for the first time in 1857 by Rudolph Albert von Kölliker as granular structures or 'sarkosomes'. In 1886 Richard Altman called them 'bioblasts' (published 1894). The word 'mitochondrium' (Greek mitos: thread; chondros: granule) was introduced by Carl Benda (1898). Mitochondria are the oxygen consuming electrochemical generators which evolved from endosymbiotic bacteria (Margulis 1970). The bioblasts of Richard Altmann (1894) include not only the mitochondria as presently defined, but also symbiotic and free-living bacteria. Mitochondria are the structural and functional elemental units of cell respiration, where cell respiration is defined as the consumption of oxygen coupled to electrochemical proton translocation across the inner mitochondrial membrane. In the process of oxidative phosphorylation (OXPHOS), the reduction of O_2 is electrochemically coupled to conservation of energy in the form of ATP (Mitchell 2011). As part of the OXPHOS system, these powerhouses of the cell contain the transmembrane respiratory complexes (i.e. FMN, Fe-S and cytochrome *b*, *c*, aa_3 redox systems), alternative dehydrogenases and oxidases, the coenzyme ubiquinone (coenzyme Q) and ATP synthase together with the enzymes of the tricarboxylic acid cycle and the fatty acid oxidation enzymes, ion transporters, including substrate, co-factor and metabolite transporters as well as proton pumps, and mitochondrial kinases related to energy transfer pathways. The mitochondrial proteome comprises over 1,200 proteins (Mitocharta), mostly encoded by nuclear DNA (nDNA), with a variety of functions, many of

which are relatively well known (*e.g.* apoptosis-regulating proteins), are still under investigation, or need to be identified (alanine transporter). Mitochondria maintain several copies of their own genome (hundred to thousands per cell) which is maternally inherited and known as mitochondrial DNA (mtDNA). mtDNA is 16.5 Kb in length, contains 13 protein-coding genes for subunits of the transmembrane respiratory Complexes CI, CIII, CIV and ATP synthase, and also encodes 22 tRNAs and the mitochondrial 16S and 12S rRNA. The mitochondrial genome is both regulated and supplemented by nuclear-encoded mitochondrial targeted proteins. Evidence has accumulated that additional gene content is encoded in the mitochondrial genome, e.g. microRNAs, piRNA, smithRNAs, repeat associated RNA, and even additional proteins. The inner mitochondrial membrane contains the non-bilayer phospholipid cardiolipin, which is not present in any other eukaryotic cellular membrane. Cardiolipin promotes the formation of respiratory supercomplexes, which are supramolecular assemblies based upon specific, though dynamic, interactions between individual respiratory complexes (Lenaz *et al.* 2017). There is a constant crosstalk between mitochondria and the other cellular components at the transcriptional or post-translational level, and through cell signalling in response to varying energy demands (Quiros *et al.* 2016). In addition to mitochondrial movement along the microtubules, mitochondrial morphology can change in response to energy requirements of the cell via processes known as fusion and fission through which mitochondria can communicate within a network, and in various pathological states which cause swelling or dysregulation of fission and fusion. Mitochondrial dysfunction is associated with a wide variety of genetic and degenerative diseases. Therefore, a better understanding of mitochondrial physiology will improve our understanding of the etiology of disease and the diagnostic repertoire of mitochondrial medicine. Abbreviation: mt, as generally used in mtDNA. Mitochondrion is singular and mitochondria is plural.

‘*For the physiologist, mitochondria afforded the first opportunity for an experimental approach to structure-function relationships, in particular those involved in active transport,*

217 *vectorial metabolism, and metabolic control mechanisms on a subcellular level*' (Ernster and
218 Schatz 1981).

219

220 **1. Introduction**

221 Mitochondria are the powerhouses of the cell with numerous physiological, molecular,
222 and genetic functions (**Box 1**). Every study of mitochondrial function and disease is faced with
223 **E**volution, **A**ge, **G**ender and sex, **L**ifestyle, and **E**nvironment (EAGLE) as essential background
224 conditions characterizing the individual patient or subject, cohort, species, tissue and to some
225 extent even cell line. As a large and highly coordinated group of laboratories and researchers,
226 the global MitoEAGLE Network's mission is to generate the necessary scale, type, and quality
227 of consistent data sets and conditions to address this intrinsic complexity. Harmonization of
228 experimental protocols and implementation of a quality control and data management system
229 is required to interrelate results gathered across a spectrum of studies and to generate a
230 rigorously monitored database focused on mitochondrial respiratory function. In this way,
231 researchers within the same and across different disciplines will be positioned to compare their
232 findings to an agreed upon set of clearly defined and accepted international standards.

233 Reliability and comparability of quantitative results depend on the accuracy of
234 measurements under strictly-defined conditions. A conceptually clearly-defined framework is
235 also required to warrant meaningful interpretation and comparability of experimental outcomes
236 carried out by research groups at different institutes. With an emphasis on quality of research,
237 collected data can be useful far beyond the specific question of a specific experiment. Vague or
238 ambiguous jargon can lead to confusion and may relegate valuable signals to wasteful noise.
239 For this reason, measured values must be expressed in standardized units for each parameter
240 used to define mitochondrial respiratory function. Standardization of nomenclature and
241 technical terms is essential to improve the awareness of the intricate meaning of divergent
242 scientific vocabulary. The 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. Coupling states of intact cells and respiratory control by fuel substrates and specific inhibitors of respiratory enzymes will be reviewed in subsequent communications.

2. 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).

Mitochondrial preparations are defined as either isolated mitochondria, or tissue and cellular preparations in which the barrier function of the plasma membrane is disrupted. The plasma membrane separates the cytosol, nucleus and organelles (the intracellular compartment) from the environment of the cell. The plasma membrane consists of a lipid bilayer, embedded proteins and attached organic molecules which collectively control the selective permeability of ions, organic molecules and particles across the cell boundary. The intact plasma membrane, therefore, prevents the passage of many water-soluble mitochondrial substrates, such as succinate or ADP, that are required for the analysis of respiratory capacity at kinetically saturating concentrations, thus limiting the scope of investigations into mitochondrial respiratory function in intact cells. The cholesterol content of the plasma membrane is high compared to mitochondrial membranes. Therefore, mild detergents, such as digitonin and saponin, can be applied to selectively permeabilize the plasma membrane by interaction with cholesterol and allow free exchange of cytosolic components with ions and organic molecules of the immediate cell environment, while maintaining the integrity and localization of organelles, cytoskeleton and the nucleus. Application of optimum concentrations of these mild

detergents leads to the complete loss of cell viability, tested by nuclear staining, while mitochondrial function remains unaffected, as shown by unaltered respiration of isolated mitochondria following addition of such low concentrations of digitonin and saponin. In addition to mechanical permeabilization during homogenization of fresh tissue, saponin may be applied additionally, to ensure permeabilization of all cells. Crude homogenate and cells permeabilized in the respiration chamber contain all components of the cell at highly diluted concentrations. All mitochondria are retained in chemically permeabilized mitochondrial preparations and crude tissue homogenates. In the preparation of isolated mitochondria the cells or tissues are homogenized, and the mitochondria are separated from other cell fractions and purified by centrifugation, entailing the loss of a significant fraction of mitochondria. The term mitochondrial preparation does not include further fractionation of mitochondrial components, as well as submitochondrial particles.

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

Coupling control states: To extend the classical nomenclature on mitochondrial coupling states (Section 2.4) 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). We focus primarily on the conceptual 'why', along with clarification of the experimental 'how'. In the following section, the concept-driven terminology is explained and coupling states are defined. The capacity of *oxidative phosphorylation*, OXPHOS, provides diagnostic reference values for physiological respiratory capacities of defined pathways of core energy metabolism and is, therefore, measured at kinetically saturating concentrations of ADP and inorganic phosphate, P_i. The *oxidative* capacity of the electron transfer system, ETS, reveals the limitation of OXPHOS capacity mediated by the *phosphorylation* system. ETS capacity is measured as noncoupled respiration by application of *external uncouplers*. The contribution of *intrinsically*

295 *uncoupled* oxygen consumption is most easily studied by not stimulating or arresting
296 phosphorylation, when oxygen consumption compensates mainly for the proton leak; the
297 corresponding states are collectively classified as LEAK states (**Table 1**). Coupling states of
298 mitochondrial preparations can be compared in any defined mitochondrial pathway control state
299 (**Fig. 1**). Fuel substrates and ETS inhibitors are kept constant while (*1*) adding ADP or P_i, (*2*)
300 inhibiting the phosphorylation system, and (*3*) performing uncoupler titrations.

301 **Respiratory capacities and kinetic control:** Coupling control states are established in
302 the study of mitochondrial preparations to obtain reference values for various output variables.
303 Physiological conditions *in vivo* may deviate substantially from these experimentally obtained
304 states. Since kinetically saturating concentrations, *e.g.* of ADP or oxygen, may not apply to
305 physiological intracellular conditions, relevant information is obtained in studies of kinetic
306 responses to conditions intermediate between the LEAK state at zero [ADP] and the OXPHOS
307 state at saturating [ADP], or of respiratory capacities in the range between kinetically saturating
308 $[O_2]$ and anoxia (Gnaiger 2001). We define respiratory capacities, comparable to channel
309 capacity in information theory, as the upper bound of the rate of respiration measured in defined
310 coupling and pathway control states of mitochondrial preparations.
311

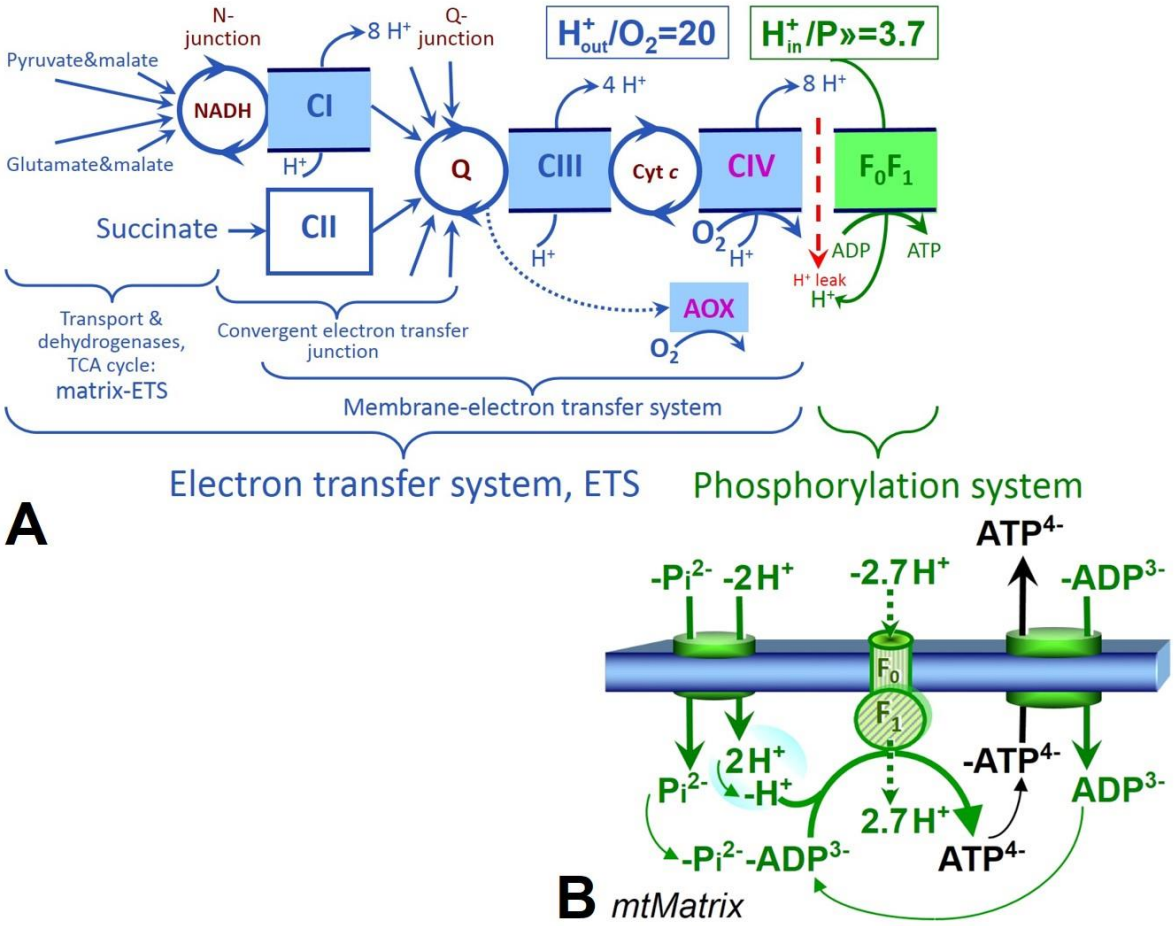


Fig. 1. The mitochondrial respiratory system and oxidative phosphorylation. (A) The electron transfer system, ETS, and coupling to the phosphorylation system. Multiple convergent electron transfer pathways are shown from NADH and succinate; additional arrows indicate electron entry through electron transferring flavoprotein, glycerophosphate dehydrogenase, dihydro-orotate dehydrogenase, choline dehydrogenase, and sulfide-ubiquinone oxidoreductase. The branched pathway of oxygen consumption by alternative quinol oxidase (AOX) is indicated by the dotted arrow. 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. $H^+_{in}/P»$ is the ratio of inward proton flux from the inter-membrane space to the flux of phosphorylation of ADP to ATP. Due to proton leak and slip these are not fixed stoichiometries. (**B**) Phosphorylation system consisting of the F_1F_0 ATP synthase, adenine nucleotide translocase, and the inorganic phosphate transporter. The $H^+_{in}/P»$ 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. 3 and 4 for further explanation. Modified from (A) Lemieux *et al*. (2017) and (B) Gnaiger (2014).

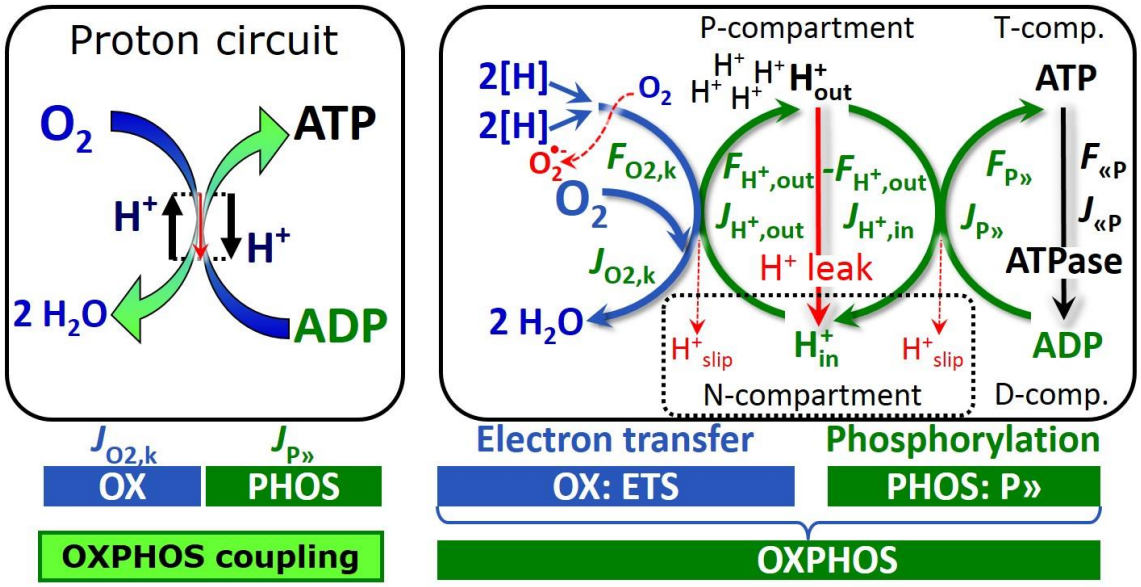


Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). Oxygen flux, $J_{O2,k}$, in a catabolic reaction k is coupled to the phosphorylation of ADP to ATP, $J_{P»}$, by the proton pumps of the electron transfer system, ETS, pushing the outward proton flux, $J_{H+,out}$, and generating the output protonmotive force, $F_{H+,out}$. ATP synthase is coupled to inward proton flux, $J_{H+,in}$, to phosphorylate ADP with inorganic phosphate to ATP, driven by the input protonmotive force, $F_{H+,in}$=-$F_{H+,out}$. 2[H] indicates the reduced hydrogen equivalents of fuel substrates that provide the chemical input force, $F_{O2,k}$ [kJ/mol O_2], of the catabolic reaction k with oxygen (Gibbs energy of reaction per mole O_2 consumed in reaction k), typically in the range of -460 to -480 kJ/mol. The output force is given by the phosphorylation potential difference (ADP phosphorylated to ATP), $F_{P»}$, which varies *in vivo* ranging from about 48 to 62 kJ/mol under physiological conditions. Fluxes, J_B, and forces, F_B, are expressed in either chemical units, [mol·s^{-1}·m^{-3}] and [J·mol^{-1}] respectively, or electrical units, [C·s^{-1}·m^{-3}] and [J·C^{-1}] respectively, per volume, V [m^3], of the system. The system defined by the boundaries shown as a full black line is not a black box, but is analysed as a compartmental system. The negative compartment (N-compartment, enclosed by the dotted line) is the matrix space, separated from the positive compartment (P-compartment) by the inner mitochondrial membrane. ADP+P_i and ATP are the substrate- and product-compartments (scalar D- and T-comp.), respectively. Chemical potentials of all substrates and products involved in the scalar reactions are measured in the P-compartment for calculation of the scalar forces $F_{O2,k}$ and $F_{P»}$=-$F_{«P}$ (**Box 2**). Modified from Gnaiger (2014).

Phosphorylation, 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 discriminating and specific 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 endergonic direction of phosphorylation ADP→ATP, and likewise the symbol «P for the corresponding exergonic hydrolysis ATP→ADP (**Fig. 2; Box 3**). ATP synthase is the proton pump of the phosphorylation system (**Fig. 1B**). P» may also involve substrate-level phosphorylation as part of the tricarboxylic acid cycle (succinyl-CoA ligase) and phosphorylation of ADP catalyzed by phosphoenolpyruvate 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 catalyzed by adenylate kinase, 2ADP ↔ ATP + AMP, proceeds without fuel substrates in the presence of ADP (Komlódi and Tretter 2017). $J_{P»}/J_{O2,k}$ (P»/O_2) is two times the 'P/O' ratio of classical bioenergetics. The effective 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 in the proton pumps when a proton effectively is not pumped; and (*4*) electron leak in the univalent reduction of oxygen (O_2; dioxygen) to superoxide anion radical ($O_2^{\bullet -}$).

Table 1. Coupling states and residual oxygen consumption in mitochondrial preparations in relation to respiration and phosphorylation rate, $J_{O2,k}$ and $J_{P»}$, and protonmotive force, $F_{H+,out}$. Coupling states are established at kinetically saturating concentrations of fuel substrates and O_2.

State	$J_{O2,k}$	$J_{P»}$	$F_{H+,out}$	Inducing factors	Limiting factors
LEAK	*L*; low proton leak-dependent respiration;	0	max.	Proton leak, slip, and cation cycling	$J_{P»}$=0: (*1*) without ADP, L_N; (*2*) max. ATP/ADP ratio, L_T; or (*3*) inhibition of the ADP phosphorylation system, L_{Omy}
OXPHOS	*P*; high ADP-stimulated respiration	max.	high	Kinetically saturating [ADP] and [P_i]	$J_{P»}$ by phosphorylation system; or $J_{O2,k}$ by electron transfer system
ETS	*E*; max. noncoupled respiration	0	low	Optimal external uncoupler concentration for max. oxygen flux	$J_{O2,k}$ by electron transfer system
ROX	*Rox*; min. residual O_2 consumption	0	0	$J_{O2,Rox}$ in non-ETS oxidation reactions	Full inhibition of ETS or absence of fuel substrates

LEAK state (Fig. 3): LEAK state is defined as 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 of *intrinsic uncoupling* without addition of any experimental uncoupler: (*1*)

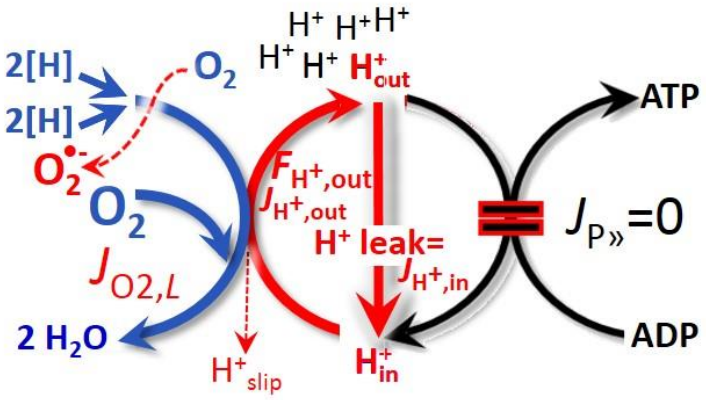


Fig. 3. LEAK state: Phosphorylation is arrested, $J_{P»}$=0, and oxygen flux, $J_{O2,L}$, is controlled mainly by the proton leak, which equals $J_{H+,in}$, at maximum protonmotive force, $F_{H+,out}$ (See also Fig. 2).

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 adenine nucleotide translocase, such as carboxyatractyloside.

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. 3**). The proton leak flux depends on the protonmotive force, is a property of the inner mitochondrial membrane, may be enhanced due to possible contaminations by free fatty acids, and is physiologically controlled. In particular, uncoupling mediated by uncoupling protein 1 (UCP1) is physiologically controlled, *e.g.*, in brown adipose tissue. UCP1 is a proton channel of the inner mitochondrial membrane facilitating the conductance of protons across the inner mitochondrial membrane. As consequence of this effective short-circuit, the protonmotive force diminishes, resulting in stimulation of electron transfer to oxygen and heat dissipation without phosphorylation of ADP. 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 in association with 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 increases with 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 mitochondrial 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.

Small differences of terms, *e.g.*, uncoupled, noncoupled, are easily overlooked and may be erroneously perceived as identical. Even with an attempt at rigorous definition, the common use of such terms may remain vague (**Table 2**).

OXPHOS state (**Fig. 4**): OXPHOS state is defined as the respiratory state with kinetically saturating concentrations of O_2, respiratory and phosphorylation substrates, and absence of exogenous uncoupler, which provides an estimate of the maximal capacity of OXPHOS in any given pathway control state.

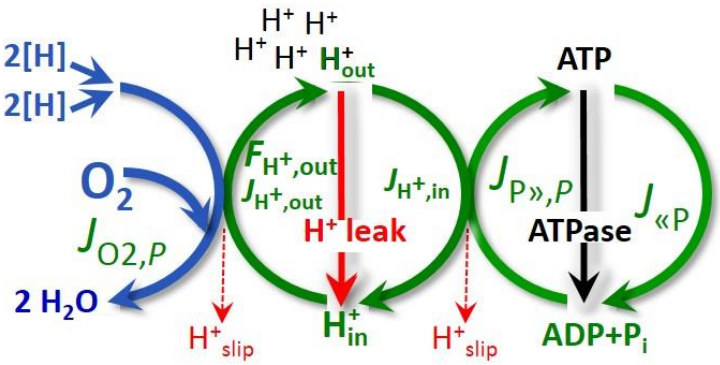


Fig. 4. OXPHOS state: Phosphorylation, $J_{P»}$, is stimulated by kinetically saturating [ADP] and inorganic phosphate, [P_i], and is supported by a high protonmotive force, $F_{H+,out}$. O_2 flux, $J_{O2,P}$, is highly coupled at a maximum P»/O_2 ratio, $J_{P»,P}/J_{O2,P}$ (See also Fig. 2).

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 capacity for oxidation during coupled respiration, 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); greater ADP concentration is required, particularly in permeabilized muscle fibres and cardiomyocytes, to overcome limitations by intracellular diffusion and by the reduced conductance of the outer mitochondrial membrane (Jepihhina *et al.* 2011, Illaste *et al.* 2012, Simson *et al.* 2016) either through interaction with tubulin (Rostovtseva *et al.* 2008) or other intracellular structures (Birkedal *et al.* 2014). 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), indicating 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 (ref). Whereas 2.5 to 5 mM ADP is sufficient to obtain the actual OXPHOS capacity in many types of permeabilized cell and tissue preparations, experimental validation is required in each specific case.

Table 2. Distinction of terms related to coupling.

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

ETS state (**Fig. 5**): 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 ETS capacity. Inhibition of

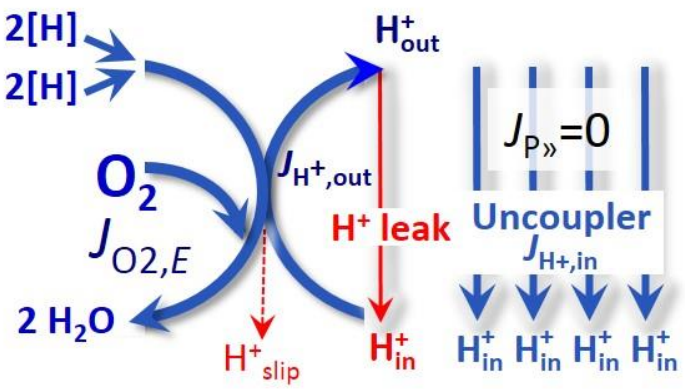


Fig. 5. ETS state: Noncoupled respiration, $J_{O2,E}$, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, $J_{P»}$=0 (See also Fig. 2).

462 respiration is observed at higher than optimum uncoupler concentrations. As a consequence of
463 the nearly collapsed protonmotive force, the driving force is insufficient for phosphorylation
464 and $J_{P»}$=0.

465 Besides the three fundamental coupling states of mitochondrial preparations, the
466 following respiratory state also is relevant to assess respiratory function:

467 **ROX:** Residual oxygen consumption (ROX) is defined as O_2 consumption due to
468 oxidative side reactions remaining after inhibition of the ETS. ROX is not a coupling state but
469 represents a baseline that is used to correct mitochondrial respiration in defined coupling states.
470 ROX is not necessarily equivalent to non-mitochondrial respiration, considering oxygen-
471 consuming reactions in mitochondria not related to ETS, such as oxygen consumption in
472 reactions catalyzed by monoamine oxidases (type A and B), monooxygenases (cytochrome
473 P450 monooxygenases), dioxygenase (sulfur dioxygenase and trimethyllysine dioxygenase),
474 several hydoxylases, and more. Mitochondrial preparations, especially those obtained from
475 liver, are contaminated by peroxisomes. This fact makes the exact determination of
476 mitochondrial oxygen consumption and mitochondria-associated generation of reactive oxygen
477 species complicated (Schönfeld *et al.* 2009). The dependence of ROX-linked oxygen
478 consumption needs to be studied in detail with respect to non-ETS enzyme activities,
479 availability of specific substrates, oxygen concentration, and electron leakage leading to the
480 formation of reactive oxygen species.

481

482 *2.2. Coupling states and respiratory rates*

483 It is important to distinguish metabolic systems from metabolic states and the
484 corresponding metabolic rates; for example: electron transfer system, ETS (**Fig. 6**), ETS state
485 (**Fig. 5**), and ETS capacity, *E*, respectively (**Table 1**). The protonmotive force is *high* in the
486 OXPHOS 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 1**).

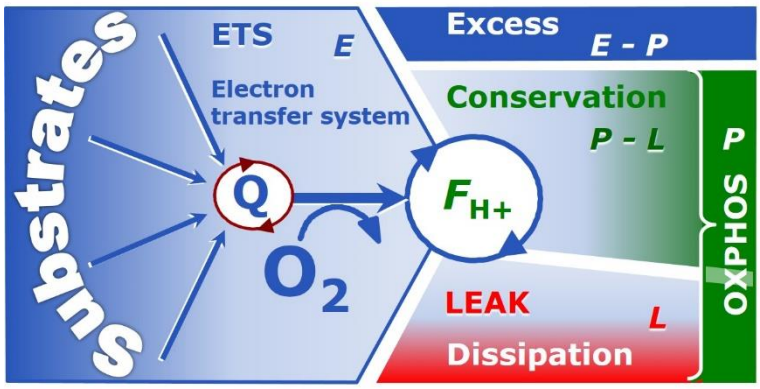


Fig. 6. Four-compartment model of oxidative phosphorylation. Respiratory states (ETS, OXPHOS, LEAK) and corresponding rates (*E, P, L*) are connected by the protonmotive force, $F_{H+,out}$. Electron transfer system capacity, *E*, is partitioned into (*1*) the dissipative LEAK respiration, *L*, when the capacity to perform work is irreversibly lost, (*2*) net OXPHOS capacity, *P-L*, with partial conservation of the capacity to perform work, and (*3*) the excess capacity, *E-P*. Modified from Gnaiger (2014).

The three coupling states, ETS, LEAK and OXPHOS, are presented in a schematic context with the corresponding respiratory rates, abbreviated as *E*, *L* and *P*, respectively (**Fig. 6**). 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*) loss of oxidative capacity during the time course of the respirometric assay, since *E* is measured subsequently to *P*; (*2*) using too low uncoupler concentrations; (*3*) using high uncoupler concentrations which inhibit the ETS (Gnaiger 2008); (*4*) high oligomycin concentrations applied for measurement of *L* before titrations of uncoupler, when oligomycin exerts an inhibitory effect on *E*. On the other hand, the excess ETS capacity is overestimated if non-saturating [P_i] or [ADP] (State 3) are used.

E>*P* is observed in many types of mitochondria, varying between species, tissues and cell types. It is the excess ETS capacity pushing the phosphorylation system (**Fig. 1B**) to the limit of its *capacity of utilizing* the protonmotive force. Within any type of mitochondria, the magnitude of *E*>*P* depends on (*1*) 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**); and (*2*) the *biochemical coupling efficiency* expressed as *(E-L)/E*, since an increase of *L* causes *P* to increase towards the limit of *E*. The *excess E-P* capacity, *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 tricarboxylic acid cycle (TCA cycle) function establish pathway control states with high ETS capacity, and consequently increase the sensitivity of the *E-P* assay.

When subtracting *L* from *P*, the dissipative LEAK component in the OXPHOS state may be overestimated. This can be avoided by measuring LEAK respiration in a state when the protonmotive force is adjusted to its slightly lower value in the OXPHOS state, *e.g.*, by titration of an ETS inhibitor. 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* or *ATP turnover* 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 freely available for ATP production (corrected for LEAK respiration) and is fully coupled to phosphorylation with a maximum mechanistic stoichiometry (**Fig. 6**).

2.3. 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 3** shows a protocol with isolated mitochondria in a closed respirometric chamber, defining a sequence of respiratory states.

Table 3. Metabolic states of mitochondria (Chance and Williams, 1956; Table V).

State	[O_2]	ADP level	Substrate level	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

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

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 3**). If addition of specific inhibitors of respiratory complexes, such as rotenone, does 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 and inhibitors. In contrast to the original definition, an alternative protocol is frequently applied, in which State 2 is induced by addition of fuel substrate without ADP (LEAK state), followed by addition of ADP.

State 3 is the state stimulated by addition of fuel substrates while the ADP concentration is still high (**Table 3**) 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. Repeated ADP titration re-establishes State 3 at ‘high ADP’. Starting at oxygen concentrations near air-saturation (ca. 200 µM O_2 at sea level and 37 °C), 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. The abbreviation State 3u is frequently used in bioenergetics, to indicate the state of respiration after titration of an uncoupler, without sufficient emphasis on the fundamental difference between OXPHOS capacity (*well-coupled* with an *endogenous* uncoupled component) and ETS capacity (*noncoupled*).

State 4 is a LEAK state which is obtained only if the mitochondrial preparation is intact and well-coupled. Depletion of ADP by phosphorylation to ATP leads to a decline in oxygen consumption in the transition from State 3 to State 4. Under these conditions, a maximum protonmotive force and high ATP/ADP ratio are maintained, and the P»/O_2 ratio can be calculated. State 4 respiration, L_T (**Table 1**), reflects intrinsic proton leak and intrinsic ATP hydrolysis activity. Oxygen consumption in State 4 is an overestimation of LEAK respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP, $J_{«P}$, which stimulates respiration coupled to phosphorylation, $J_{P»}>0$. This can be tested by inhibition of the phosphorylation system using oligomycin, ensuring that $J_{P»}=0$ (State 4o). Alternatively, sequential ADP titrations re-establish State 3, followed by State 3 to State 4 transitions while sufficient oxygen is available. However, anoxia may be reached before exhaustion of ADP (State 5).

State 5 is the state 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).

In **Table 3**, only States 3 and 4 (and ‘State 2’ in the alternative protocol without ADP; not included in the table) are coupling control states, with the restriction that O_2 flux in State 3 may be limited kinetically by non-saturating ADP concentrations (**Table 1**).

3. The protonmotive force and proton flux

3.1. Electric and chemical partial forces versus electrical and chemical units

The protonmotive force across the inner mitochondrial membrane (Mitchell and Moyle 1967) was introduced most beautifully in the *Grey Book 1966* (see Mitchell 2011),

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

The protonmotive force consists of two partial forces: (*1*) The electrical part, $\Delta\Psi$, is the difference of charge (electric potential difference) and is not specific for H^+. (*2*) The chemical part, $\Delta\mu_{H+}$, is the chemical potential difference in H^+, is proportional to the pH difference, and incorporates the Faraday constant (**Table 4**).

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

State		Force		electric	+	chem.	Unit	Notes
	Protonmotive force, *e*	Δp_{H+}	=	$\Delta\Psi$	+	$\Delta\mu_{H+}/F$	$J{\cdot}C^{-1}$	1*e*
	Chemiosmotic potential, *n*	$\Delta\tilde{\mu}_{H+}$	=	$\Delta\Psi{\cdot}F$	+	$\Delta\mu_{H+}$	$J{\cdot}mol^{-1}$	1*n*
State	**Isomorphic force**	$F_{H+,out/i}$		**el_{out}**	+	**$H^+_{out,d}$**		
	Electric charge, *e*	$F_{H+,out/e}$	=	$F_{el,out/e}$	+	$F_{H+,out,d/e}$	$J{\cdot}C^{-1}$	2*e*
	Amount of substance, *n*	$F_{H+,out/n}$	=	$F_{el,out/n}$	+	$F_{H+,out,d/n}$	$J{\cdot}mol^{-1}$	2*n*
Rate	**Isomorphic flux**	$J_{H+,out/i}$		***e***	**or**	***n***		
	Electric charge, *e*	$J_{H+,out/e}$		$J_{H+,out/e}$			$C{\cdot}s^{-1}{\cdot}m^{-3}$	3*e*
	Amount of substance, *n*	$J_{H+,out/n}$				$J_{H+,out/n}$	$mol{\cdot}s^{-1}{\cdot}m^{-3}$	3*n*

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

2: The protonmotive force is $F_{H+,out}$, expressed either in isomorphic format *e* or *n*. $F_{el/e} \equiv \Delta\Psi$ is the partial protonmotive force (el) acting generally on charged motive molecules (*i.e.* ions that are displaceable across the inner mitochondrial membrane). In contrast, $F_{H+,d/n} \equiv \Delta\mu_{H+}$ is the partial protonmotive force specific for proton displacement (H^+_d). The sign of the force is negative for exergonic transformations in which exergy is lost or dissipated, and positive for endergonic transformations which conserve exergy from a coupled exergonic process (**Box 3**).

3: The sign of the flux depends on the definition of the compartmental direction of the translocation (**Fig. 2**). Flux x force = $J_{H+,out/e} \cdot F_{H+,out/e} = J_{H+,out/n} \cdot F_{H+,out/n}$ = Volume-specific power [$J \cdot s^{-1} \cdot m^{-3} = W \cdot m^{-3}$].

Faraday constant, $F = eN_A$ [C/mol] (**Table 4**), enables the conversion between protonmotive force, $F_{H+,out/e} \equiv \Delta p_{H+}$ [J/C], expressed per *motive charge*, *e* [C], and protonmotive force or electrochemical potential difference, $F_{H+,out/n} \equiv \Delta\tilde{\mu}_{H+} = \Delta p_{H+} \cdot F$ [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* converts protonmotive force and flux from isomorphic format *e* to *n* (Eq. 2; see also **Table 4**, Note 2),

$$F_{H+,out/n} = F_{H+,out/e} \cdot eN_A \qquad \text{(Eq. 2.1)}$$

$$J_{H+,out/n} = J_{H+,out/e} / (eN_A) \qquad \text{(Eq. 2.2)}$$

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

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

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

Protonmotive means that there is a potential for the movement of protons, and force is a measure of the potential for motion. Motion is relative and not absolute (Principle of Galilean Relativity); likewise there is no absolute potential, but (isomorphic) forces are potential differences. An electric partial force expressed in the format of electric charge, $F_{el/e}$, of -0.2 V (**Table 5**, Note 5*e*) is equivalent to force in the format of amount, $F_{el,H+/n}$, of 19 $kJ \cdot mol^{-1}$ H^+_{out} (Note 5*n*). For a ΔpH of 1 unit, the chemical partial force in the format of amount, $F_{d,H+/n}$, changes by 5.9 $kJ \cdot mol^{-1}$ (**Table 5**, Note 6*n*) and chemical force in the format of charge $F_{d,H+/e}$ changes by 0.06 V (Note 6*e*). Considering a driving force of -470 $kJ \cdot mol^{-1}$ O_2 for oxidation, the thermodynamic limit of the H^+_{out}/O_2 ratio is reached at a value of 470/19=24, compared to a mechanistic stoichiometry of 20 (**Fig. 1**).

3.2. Definitions

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 induced by experimental control signals that *exert* an influence on: (*1*) ATP demand and ADP phosphorylation rate; (*2*) fuel substrate composition, pathway competition; (*3*) available amounts of substrates and oxygen,

e.g., starvation and hypoxia; (*3*) the protonmotive force, redox states, flux-force relationships, coupling and efficiency; (*4*) Ca^{2+} and other ions including H^+; (*5*) inhibitors, *e.g.*, nitric oxide or intermediary metabolites, such as oxaloacetate; (*6*) signalling pathways and regulatory proteins, *e.g.* insulin resistance, transcription factor HIF-1 or inhibitory factor 1. *Mechanisms* of respiratory control and regulation include adjustments of (*1*) enzyme activities by allosteric mechanisms and phosphorylation, (*2*) enzyme content, concentrations of cofactors and conserved moieties (such as adenylates, nicotinamide adenine dinucleotide [NAD^+/NADH], coenzyme Q, cytochrome *c*); (*3*) metabolic channeling by supercomplexes; and *(4)* mitochondrial density (enzyme concentrations and membrane area) and morphology (cristae folding, fission and fusion). (*5*) Mitochondria are targeted directly by hormones, thereby affecting their energy metabolism (Lee *et al.* 2013; Gerö and Szabo 2016; Price and Dai 2016; Moreno *et al.* 2017). Evolutionary or acquired differences in the genetic and epigenetic basis of mitochondrial function (or dysfunction) between subjects and gene therapy; age; gender, biological sex, and hormone concentrations; life style including exercise and nutrition; and environmental issues including thermal, atmospheric, toxicological and pharmacological factors, exert an influence on all control mechanisms listed above (for reviews, see Brown 1992; Gnaiger 1993a, 2009; 2014; Paradies *et al.* 2014; Morrow *et al*. 2017).

Respiratory control and response: Lack of control by a metabolic system, *e.g.* phosphorylation system, does mean that there will be no response to a variable activating it, *e.g.* [ADP]. However, the reverse is not true as the absence of a response to [ADP] does not exclude the phosphorylation system from 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 or proton leak flux (**Box 2**). As such, it is necessary to be specific as to which input and output are under consideration (Fell 1997). Therefore, the term respiratory control is elaborated in more detail in the following section.

689 **Respiratory coupling control:** Respiratory control refers to the ability of mitochondria
690 to adjust oxygen consumption in response to external control signals by engaging various
691 mechanisms of control and regulation. Respiratory control is monitored in a mitochondrial
692 preparation under conditions defined as respiratory states. When phosphorylation of ADP to
693 ATP is stimulated or depressed, an increase or decrease is observed in electron flux linked to
694 oxygen consumption in respiratory coupling states of intact mitochondria ('controlled states' in
695 the classical terminology of bioenergetics). Alternatively, coupling of electron transfer with
696 phosphorylation is disengaged by disruption of the integrity of the inner mitochondrial
697 membrane or by uncouplers, functioning like a clutch in a mechanical system. The
698 corresponding coupling control state is characterized by high levels of oxygen consumption
699 without control by phosphorylation ('uncontrolled state'). Energetic coupling is defined in **Box**
700 **4**. Loss of coupling by intrinsic uncoupling and decoupling, or pathological dyscoupling lowers
701 the efficiency. Such generalized uncoupling is different from switching to mitochondrial
702 pathways that involve fewer than three proton pumps ('coupling sites': Complexes CI, CIII and
703 CIV), bypassing CI through multiple electron entries into the Q-junction (**Fig. 1**). A bypass of
704 CIII and CIV is provided by alternative oxidases, which reduce oxygen without proton
705 translocation. Reprogramming of mitochondrial pathways may be considered as a switch of
706 gears (changing the stoichiometry) rather than uncoupling (loosening the stoichiometry).
707 **Pathway control states** are obtained in mitochondrial preparations by depletion of
708 endogenous substrates and addition to the mitochondrial respiration medium of fuel substrates
709 (CHNO) and specific inhibitors, activating selected mitochondrial pathways (**Fig. 1**). Coupling
710 control states and pathway control states are complementary, since mitochondrial preparations
711 depend on an exogenous supply of pathway-specific fuel substrates and oxygen (Gnaiger 2014).
712
713
714

Box 2: Metabolic fluxes and flows: vectorial and scalar

In the mitochondrial electron transfer system (**Fig. 1**), vectorial transmembrane proton flux is coupled through the proton pumps CI, CIII and CIV to the catabolic flux of scalar reactions, collectively measured as oxygen flux. In **Fig. 2**, the scalar catabolic reaction, k, of oxygen consumption, $J_{O2,k}$ [mol·s^{-1}·m^{-3}], is expressed as oxygen flux per volume, V [m^3], of the instrumental chamber (the system).

Fluxes are *vectors*, if they have *spatial* direction in addition to magnitude. A vector flux (surface-density of flow) is expressed per unit cross-sectional area, A [m^2], perpendicular to the direction of flux. If *flows*, I, are defined as extensive quantities of the *system*, as vector or scalar flow, $\boldsymbol{I}$ or I [mol·s^{-1}], respectively, then the corresponding vector and scalar *fluxes*, J, are obtained as $\boldsymbol{J}=\boldsymbol{I}\cdot A^{-1}$ [mol·s^{-1}·m^{-2}] and $J=I\cdot V^{-1}$ [mol·s^{-1}·m^{-3}], respectively, expressing flux as an area-specific vector or volume-specific scalar quantity.

Vectorial transmembrane proton flux, $J_{H+,out}$, is analyzed in a heterogenous compartmental system as a quantity with *directional* but not *spatial* information. Translocation of protons across the inner mitochondrial membrane has a defined direction, either from the negative compartment (matrix space; N-phase) to the positive compartment (inter-membrane space; P-phase) or *vice versa* (**Fig. 2**). The arrows defining the direction of the translocation between the two compartments may point upwards or downwards, right or left, without any implication that these are actual directions in space. The 'upper' compartment of the P-phase is neither above nor below the N-phase in a spatial sense, but can be visualized arbitrarily in a figure as the upper compartment (**Fig. 2**). In general, the *compartmental direction* of vectorial translocation from the N-phase to the P-phase is defined by assigning the initial and final state as *ergodynamic compartments*, $H^+_{in} \rightarrow H^+_{out}$, respectively, related to work (erg = work) that must be performed to lift the proton from a lower to a higher electrochemical potential or from the lower to the higher ergodynamic compartment (Gnaiger 1993b).

In direct analogy to *vectorial* translocation, the direction of a *scalar* chemical reaction, A → B, is defined by assigning substrates and products, A and B, as ergodynamic compartments. O_2 is defined as a substrate in respiratory O_2 consumption, which together with the fuel substrates comprises the substrate compartment of the catabolic reaction (**Fig. 2**). Volume-specific scalar O_2 flux is coupled (**Box 4**) to vectorial translocation. In order to establish a quantitative relation between the coupled fluxes, both $J_{O2,k}$ and $J_{H+,out}$ must be expressed in identical units ([$mol \cdot s^{-1} \cdot m^{-3}$] or [$C \cdot s^{-1} \cdot m^{-3}$]), yielding the H^+_{out}/O_2 ratio (**Fig. 1**). The *vectorial* proton flux in compartmental translocation has *compartmental direction*, distinguished from a *vector* flux with *spatial direction*. Likewise, the corresponding protonmotive force is defined as an electrochemical potential *difference* between two compartments, in contrast to a *gradient* across the membrane or a vector force with defined spatial direction.

The steady-state: Mitochondria represent a thermodynamically open system functioning as a biochemical transformation system in non-equilibrium states. State variables (protonmotive force; 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 for by *external* fluxes *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 O_2, fuel substrates, ADP, P_i, H^+) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require respiratory media with sufficient buffering capacity and kinetically saturating concentrations of substrates to be maintained, and thus depend on the kinetics of the processes under investigation. Proton turnover, $J_{\infty H+}$, and ATP turnover, $J_{\infty P}$, proceed in the steady-state at constant $F_{H+,out}$, when $J_{\infty H+} = J_{H+,out} = J_{H+,in}$, and at constant $F_{P»}$, when $J_{\infty P} = J_{P»} = J_{«P}$ (**Fig. 2**).

Box 3: Endergonic and exergonic transformations, exergy and dissipation

A chemical reaction, or any transformation, is exergonic if the Gibbs energy change (exergy) of the reaction is negative at constant temperature and pressure. The sum of Gibbs energy changes of all internal transformations in a system can only be negative, i.e. exergy is irreversibly dissipated. Endergonic reactions are characterized by positive Gibbs energies of reaction and cannot proceed spontaneously in the forward direction as defined. For instance, the endergonic reaction P» is coupled to exergonic catabolic reactions, such that the total Gibbs energy change is negative, *i.e.* exergy must be dissipated for the reaction to proceed (**Fig. 2**).

In contrast, energy cannot be lost or produced in any internal process, which is the key message of the first law of thermodynamics. Thus mitochondria are the sites of energy transformation but not energy production. Open and closed systems can gain energy and exergy only by external fluxes, *i.e.* uptake from the environment. Exergy is the potential to perform work. In the framework of flux-force relationships (**Box 4**), the *partial* derivative of Gibbs energy per advancement of a transformation is an isomorphic force, F_{tr} (**Table 5**, Note 2). In other words, force is equal to exergy/motive unit (in integral form, this definition takes care of non-isothermal processes). 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).

Table 5. Power, exergy, force, flux, and advancement.

Expression	Symbol	Definition	Unit	Notes
Power, volume-specific	$P_{V,tr}$	$P_{V,tr} = J_{tr} \cdot F_{tr} = \partial_{tr}G \cdot \partial t^{-1}$	$W=J \cdot s^{-1} \cdot m^{-3}$	1
Force, isomorphic	F_{tr}	$F_{tr} = \partial_{tr}G \cdot \partial_{tr}\xi^{-1}$	$J \cdot x^{-1}$	2
Flux, isomorphic	J_{tr}	$J_{tr} = d_{tr}\xi \cdot dt^{-1} \cdot V^{-1}$	$x \cdot s^{-1} \cdot m^{-3}$	3
Advancement, *n*	$d_{tr}\xi_{H+/n}$	$d_{tr}\xi_{H+/n} = d_{tr}n_{H+} \cdot \nu_{H+}^{-1}$	Mol	4*n*
Advancement, *e*	$d_{tr}\xi_{H+/e}$	$d_{tr}\xi_{H+/e} = d_{tr}e_{H+} \cdot \nu_{H+}^{-1}$	C	4*e*
Electric partial force, *e*	$F_{el/e}$	$F_{el/e} \equiv \Delta\Psi$	V	5*e*
Electric partial force, *n*	$F_{el/n}$	$\Delta\Psi \cdot F = 96.5 \cdot \Delta\Psi$	$kJ \cdot mol^{-1}$	5*n*
Chemical partial force, *e*	$F_{d,H+/e}$	$\Delta\mu_{H+}/F = -\ln(10) \cdot RT/F \cdot \Delta pH$	V	6*e*
at 37 °C		$= -0.06 \cdot \Delta pH$	$J \cdot C^{-1}$	
Chemical partial force, *n*	$F_{d,H+/n}$	$\Delta\mu_{H+} = -\ln(10) \cdot RT \cdot \Delta pH$	$J \cdot mol^{-1}$	6*n*
at 37 °C		$= -5.9 \cdot \Delta pH$	$kJ \cdot mol^{-1}$	

1 to 4: An isomorphic motive entity or transformant, expressed in units x, is defined for any transformation, tr. x=mol or C in proton translocation.

2: $\partial_{tr}G$ [J] is the partial Gibbs energy change in the advancement of transformation tr.

3: For x=C, flow is electric current, I_{el} [A = $C \cdot s^{-1}$], vector flux is electric current density per area, $\boldsymbol{J}_{el}$, and compartmental flux is electric current density per volume, I_{el} [$A \cdot m^{-3}$].

4*n*: For a chemical reaction, the advancement of reaction r is $d_r\xi_B = d_r n_B \cdot \nu_B^{-1}$ [mol]. The stoichiometric number is ν_B=-1 or ν_B=1, depending on B being a product or substrate, respectively, in reaction r involving one mole of B. The conjugated *intensive* molar quantity, $F_{B,r} = \partial_r G/\partial_r \xi_B$ [$J \cdot mol^{-1}$], 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 for 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.

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

5*n*: F=96.5 (kJ·mol^{-1})/V.

6: The electric partial force is independent of temperature (Note 5), but the chemical partial force depends on absolute temperature, T [K].

6*e*: RT is the gas constant times absolute temperature. ln(10)·RT/F = 59.16 and 61.54 mV at 298.15 and 310.15 K (25 and 37 °C), respectively.

6*n*: ln(10)·RT = 5.708 and 5.938 kJ·mol^{-1} at 298.15 and 310.15 K (25 and 37 °C), respectively.

3.3. Forces and fluxes in physics and irreversible thermodynamics

According to its definition in physics, a potential difference and as such the *protonmotive force*, Δp_{H+}, is not a force *per se* (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' *flux-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 derivative of potentially available or 'free' energy (exergy) per isomorphic *motive* unit (**Box 3**). 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 (**Box 2**). 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: 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 (**Box 4**). 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 approaches zero at the maximum efficiency of 1, and the process becomes fully reversible without any dissipation of exergy, i.e. without entropy production.

Box 4: Coupling, power and efficiency, at constant temperature and pressure

Energetic coupling means that two processes of energy transformation are linked such that the input power, P_{in}, is the driving element of the output power, P_{out}, and the out/input power ratio is the efficiency. In general, power is work per unit time [$J.s^{-1}$=W]. When describing a system with volume V without information on the internal structure, the output is defined as the *external* work (exergy) performed by the *total* system on its environment. Such a system may be open for any type of exchange, or closed and thus allowing only heat and work to be exchanged across the system boundaries. This is the classical black box approach of thermodynamics. In contrast, in a colourful compartmental analysis of *internal* energy transformations (**Fig. 2**), the system is structured and described by definition of ergodynamic compartments (with information on the heterogeneity of the system; **Box 2**) and analysis of separate parts, *i.e.* a sequence of *partial* energy transformations, tr. In general, power per unit volume, P_{tr}/V [$W.L^{-1}$], is the product of a volume-specific flux, J_{tr}, and its conjugated force, F_{tr}, and is closely linked

to the dissipation function using the terminology of irreversible thermodynamics (Prigogine 1967; Gnaiger 1993a,b). Output power of proton translocation and catabolic input power are (**Fig. 2**),

Output: $$P_{H+,out}/V = J_{H+,out} \cdot F_{H+,out}$$

Input: $$P_k/V = J_{O2,k} \cdot F_{O2,k}$$

$F_{O2,k}$ is the exergonic input force with a negative sign, and, $F_{H+,out}$, is the endergonic output force with a positive sign (**Box 3**). Ergodynamic efficiency is the ratio of output/input power, or the flux ratio times force ratio (Gnaiger 1993a,b),

$$\varepsilon = \frac{P_{H+,out}}{-P_k} = \frac{J_{H+,out}}{J_{O2,k}} \cdot \frac{F_{H+,out}}{-F_{O2,k}}$$

The concept of incomplete coupling relates exclusively to the first term, *i.e.* the flux ratio, or H^+_{out}/O_2 ratio (**Fig. 1**). Likewise, respirometric definitions of the P»/O_2 ratio and biochemical coupling efficiency (Section 3.2) consider flux ratios. In a completely coupled process, the power efficiency, ε, depends entirely on the force ratio, ranging from zero efficiency at an output force of zero, to the limiting output force and maximum efficiency of 1.0, when the total power of the coupled process, $P_t=P_k+P_{H+,out}$, equals zero, and any net flows are zero at ergodynamic equilibrium of a coupled process. Thermodynamic equilibrium is defined as the state when all potentials (all forces) are dissipated and equilibrate towards their minima of zero. In a fully or completely coupled process, output and input fluxes are directly proportional in a fixed ratio technically defined as a stoichiometric relationship (a gear ratio in a mechanical system). Such maximal stoichiometric output/input flux ratios are considered in OXPHOS analysis as the upper limits or mechanistic H^+_{out}/O_2 and P»/O_2 ratios (**Fig. 1**).

Coupled versus bound processes: Since the chemiosmotic theory describes the mechanisms of coupling in OXPHOS, it may be interesting to ask if the electrical 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 electrical and chemical processes, which are only *theoretically* partitioned as electrical 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 electrical and chemical parts are tightly bound partial forces of the protonmotive force, since a flux cannot be partitioned but expressed only in either an electrical or chemical isomorphic format (**Table 4**).

4. Normalization: fluxes and flows

The challenges of measuring mitochondrial respiratory flux are matched by those of normalization, whereby O_2 consumption may be considered as the nominator and normalization as the complementary denominator, which are tightly linked in reporting the measurements in a format commensurate with the requirements of a database.

4.1. Flux per chamber volume

The volume-specific *flux of a chemical reaction* r is the time derivative of the advancement of the reaction per unit volume, $J_{V,B} = d_r\xi_B/dt \cdot V^{-1}$ [$(mol \cdot s^{-1}) \cdot L^{-1}$]. The *rate of concentration change* is dc_B/dt [$(mol \cdot L^{-1}) \cdot s^{-1}$], where concentration is $c_B = n_B/V$. It is helpful to make the subtle distinction between [$mol \cdot s^{-1} \cdot L^{-1}$] and [$mol \cdot L^{-1} \cdot s^{-1}$] for the fundamentally different quantities of volume-specific flux and rate of concentration change, which merge to a single expression only in closed systems. In open systems, external fluxes (such as O_2 supply) are distinguished from internal transformations (metabolic flux, O_2 consumption). In a closed system, external flows of all substances are zero and O_2 consumption (internal flow), I_{O2} [$pmol \cdot s^{-1}$], causes a decline of the amount of O_2 in the system, n_{O2} [nmol]. Normalization of these quantities for the volume of the system, V [$L = dm^3$], yields volume-specific O_2 flux,

917 $J_{V,O2}=I_{O2}/V$ [nmol·s^{-1}·L^{-1}], and O_2 concentration, [O_2] or $c_{O2}=n_{O2}/V$ [nmol·mL^{-1}=µmol·L^{-1}=µM].
918 Instrumental background O_2 flux is due to external flux into a non-ideal closed respirometer,
919 such that total volume-specific flux has to be corrected for instrumental background O_2 flux,
920 i.e. O_2 diffusion into or out of the instrumental chamber. $J_{V,O2}$ is relevant mainly for
921 methodological reasons and should be compared with the accuracy of instrumental resolution
922 of background-corrected flux, *e.g.* ±1 nmol·s^{-1}·L^{-1} (Gnaiger 2001). 'Metabolic' or catabolic
923 indicates O_2 flux, $J_{O2,k}$, corrected for instrumental background O_2 flux and chemical background
924 O_2 flux due to autoxidation of chemical components added to the incubation medium.
925
926 *4.2. System-specific and sample-specific normalization*
927 Application of common and generally defined units is required for direct transfer of
928 reported results into a database. The second [s] is the *SI* unit for the base quantity *time*. It is also
929 the standard time-unit used in solution chemical kinetics. **Table 6** lists some conversion factors
930 to obtain *SI* units. The term *rate* is not sufficiently defined to be useful for a database (**Fig. 7**).
931 The inconsistency of the meanings of rate becomes fully apparent when considering Galileo
932 Galilei's famous principle, that 'bodies of different weight all fall at the same rate (have a
933 constant acceleration)' (Coopersmith 2010).
934 **Extensive quantities:** An extensive quantity increases proportionally with system size.
935 The magnitude of an extensive quantity is completely additive for non-interacting subsystems,
936 such as mass or flow expressed per defined system. The magnitude of these quantities depends
937 on the extent or size of the system (Cohen *et al.* 2008).

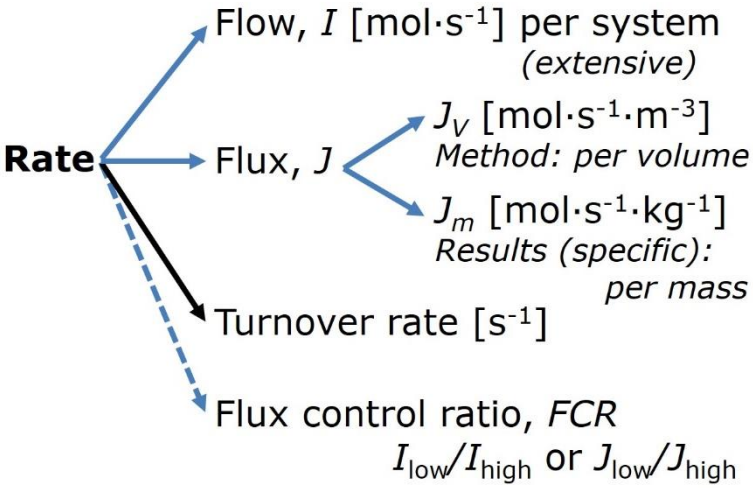


Fig. 7. Different meanings of *rate* may lead to confusion, if the normalization is not sufficiently specified. Results are frequently expressed as mass-specific *flux*, J_m, per mg protein, dry or wet weight (mass). Cell volume, V_{cell}, or mitochondrial volume, V_{mt}, may be used for normalization (volume-specific flux, J_{Vcell} or J_{Vmt}), which then must be clearly distinguished from flux, J_V, expressed for methodological reasons per volume of the measurement system, or flow per cell, I_X.

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). Mass-specific flux is flow divided by mass of the system. A mass-specific quantity 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 further clarified, 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 *in a system* or for amount of motive substance *in a transformation*. 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, $G_B = G/n_B$ [J·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), $\partial_r\xi_B$ [mol], the resulting intensive molar quantity, $F_{r,B} = \partial G/\partial_r\xi_B$ [J·mol^{-1}], is the chemical motive force of reaction r involving 1 mol B (**Table 5**, Note 4).

Flow per system, *I*: In analogy to electrical 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} [A=C·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), $\boldsymbol{J}_{el}$ [A·m^{-2} = C·s^{-1}·m^{-2}].

Size-specific flux, *J*: Metabolic O_2 flow per tissue increases as tissue mass is increased. Tissue mass-specific O_2 flux should be independent of the size of the tissue sample studied in the instrument chamber, but volume-specific O_2 flux (per volume of the instrument chamber, *V*) should increase in direct proportion to the amount of sample in the chamber. Accurate definition of the experimental system is decisive: whether 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 O_2 flux depends on mass-concentration of the sample in the chamber, but should be independent of the chamber volume. There are practical limitations to increasing the mass-concentration of the sample in the chamber, when one is concerned about crowding effects and instrumental time resolution.

Sample concentration C_{mX}: Normalization for sample concentration is required for reporting respiratory data. Consider a tissue or cells as the sample, *X*, and the sample mass, m_X [mg] 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 [mg]), or as amount of tissue or cell protein ($m_X \equiv m_{Protein}$). In the case of permeabilized tissues, cells, and homogenates, the sample concentration, $C_{mX}=m_X/V$ [mg·mL^{-1}=g·L^{-1}], is simply the mass of the subsample of tissue that is transferred into the instrument chamber. Part of the mitochondria from the tissue is 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 in preparations characterized by low mitochondrial yield. Determination of the mitochondrial yield is based on measurement of the concentration of a mitochondrial marker in the tissue homogenate, $C_{\mathrm{mte,thom}}$, which simultaneously provides information on the specific mitochondrial density in the sample (**Fig. 8**).

Tissues can contain multiple cell populations which may have distinct mitochondrial subtypes. Mitochondria are also in a constant state of flux due to highly dynamic fission and fusion cycles, and can exist in multiple stages and sizes which may be altered by a range of factors. The isolation of mitochondria (often achieved through differential centrifugation) can therefore yield a subsample of the mitochondrial types present in a tissue, dependent on isolation protocols utilized (e.g. centrifugation speed). This possible artefact should be taken into account when planning experiments using isolated mitochondria. The tendency for mitochondria of specific sizes to be enriched at different centrifugation speeds also has the potential to allow the isolation of specific mitochondrial subpopulations and therefore the analysis of mitochondria from multiple cell lineages within a single tissue.

Mass-specific flux, $J_{mX,O2}$: Mass-specific flux is obtained by expressing respiration per mass of sample, m_X [mg]. X is the type of sample, *e.g.*, tissue homogenate, permeabilized fibres or cells. Volume-specific flux is divided by mass concentration of X, $J_{mX,O2} = J_{V,O2}/C_{mX}$; or flow per cell is divided by mass per cell, $J_{m\mathrm{cell,O2}} = I_{\mathrm{cell,O2}}/M_{\mathrm{cell}}$. 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 respires 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. Optimization of cell density and arrangement is generally important and particularly in

experiments carried out in wells, considering the confluency of the cell monolayer or clumps of cells (Salabei *et al.* 2014).

Table 6. Sample concentrations and normalization of flux with *SI* base units.

Expression	Symbol	Definition	*SI* Unit	Notes
Sample				
Identity of sample	X	Cells, animals, patients		
Number of sample entities X	N_X	Number of cells, *etc.*	x	
Mass of sample X	m_X		kg	1
Mass of entity X	M_X	$M_X = m_X \cdot N_X^{-1}$	$kg \cdot x^{-1}$	1
Mitochondria				
Mitochondria	mt	X=mt		
Amount of mt-elements	mte	Quantity of mt-marker	x_{mte}	
Concentrations				
Sample number concentration	C_{NX}	$C_{NX} = N_X \cdot V^{-1}$	$x \cdot m^{-3}$	2
Sample mass concentration	C_{mX}	$C_{mX} = m_X \cdot V^{-1}$	$kg \cdot m^{-3}$	
Mitochondrial concentration	C_{mte}	$C_{mte} = mte \cdot V^{-1}$	$x_{mte} \cdot m^{-3}$	3
Specific mitochondrial density	D_{mte}	$D_{mte} = mte \cdot m_X^{-1}$	$x_{mte} \cdot kg^{-1}$	4
Mitochondrial content, mte per entity X	mte_X	$mte_X = mte \cdot N_X^{-1}$	$x_{mte} \cdot x^{-1}$	5
O_2 flow and flux				6
Flow	I_{O2}	Internal flow	$mol \cdot s^{-1}$	7
Volume-specific flux	$J_{V,O2}$	$J_{V,O2} = I_{O2} \cdot V^{-1}$	$mol \cdot s^{-1} \cdot m^{-3}$	8
Flow per sample entity X	$I_{X,O2}$	$I_{X,O2} = J_{V,O2} \cdot C_{NX}^{-1}$	$mol \cdot s^{-1} \cdot x^{-1}$	9
Mass-specific flux	$J_{mX,O2}$	$J_{mX,O2} = J_{V,O2} \cdot C_{mX}^{-1}$	$mol \cdot s^{-1} \cdot kg^{-1}$	
Mitochondria-specific flux	$J_{mte,O2}$	$J_{mte,O2} = J_{V,O2} \cdot C_{mte}^{-1}$	$mol \cdot s^{-1} \cdot x_{mte}^{-1}$	10

1 The *SI* prefix k is used for the SI base unit of mass (kg=1,000 g). In praxis, various *SI* prefixes are used for convenience, to make numbers easily readable, *e.g.* 1 mg tissue, cell or mitochondrial mass instead of 0.000001 kg.

2 In case X=cells, the sample number concentration is $C_{Ncell}=N_{cell} \cdot V^{-1}$, and volume may be expressed in [dm^3=L] or [cm^3=mL]. See Table 7 for different sample types.

3 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*) $C_{mte}=C_{mX} \cdot D_{mte}$.

4 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.

5 $mte_X = mte \cdot N_X^{-1} = C_{mte} \cdot C_{NX}^{-1}$.

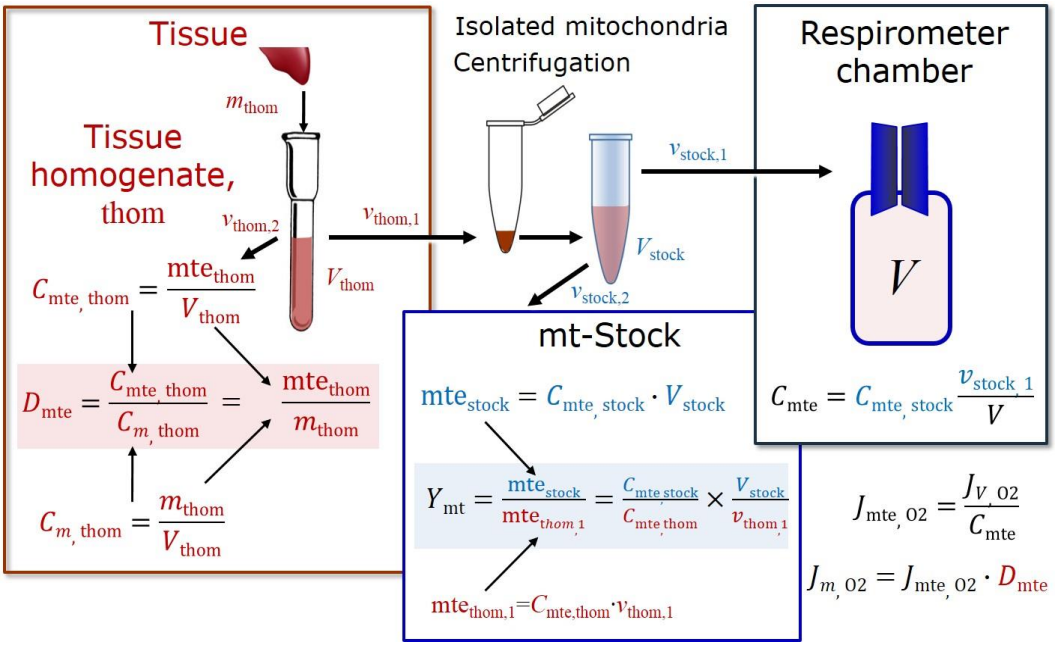
6 Entity O_2 can be replaced by other chemical entities B to study different reactions.

7 I_{O2} and V are defined per instrument chamber as a system of constant volume (and constant temperature), which may be closed or open. I_{O2} is abbreviated for $I_{O2,r}$, *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, $\nu_{O2}=-1$. $I_{O2,r} = d_r n_{O2}/dt \cdot \nu_{O2}^{-1}$. If r includes all chemical reactions in which O_2 participates, then $d_r n_{O2} = dn_{O2} - d_e n_{O2}$, where dn_{O2} is the change in the amount of O_2 in the instrument chamber and $d_e n_{O2}$ is the amount of O_2 added externally to the system. At steady state, by definition $dn_{O2}=0$, hence $d_r n_{O2} = -d_e n_{O2}$.

8 $J_{V,O2}$ is an experimental variable, expressed per volume of the instrument chamber.

9 $I_{X,O2}$ is a physiological variable, depending on the size of entity X.

10 There are many ways to normalize for a mitochondrial marker, that are used in different experimental approaches: (*1*) $J_{mte,O2} = J_{V,O2} \cdot C_{mte}^{-1}$; (*2*) $J_{mte,O2} = J_{V,O2} \cdot C_{mX}^{-1} \cdot D_{mte}^{-1} = J_{mX,O2} \cdot D_{mte}^{-1}$; (*3*) $J_{mte,O2} = J_{V,O2} \cdot C_{NX}^{-1} \cdot mte_X^{-1} = I_{X,O2} \cdot mte_X^{-1}$; (*4*) $J_{mte,O2} = I_{O2} \cdot mte^{-1}$.



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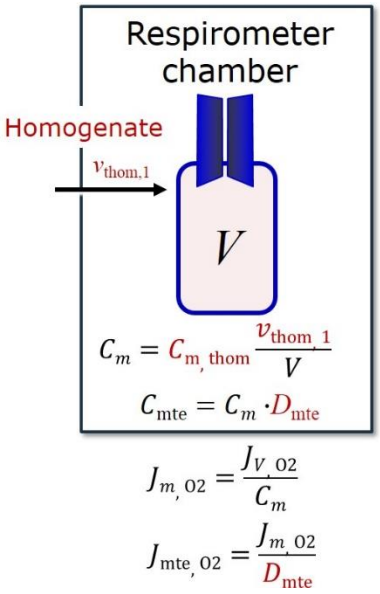
Symbol	Definition [Units]
C_{mte}	Mitochondrial concentration in chamber [$x_{mte} \cdot L^{-1}$]
C_m	Sample mass concentration in chamber [$g \cdot L^{-1}$]
D_{mte}	Specific mte-density per tissue mass [$x_{mte} \cdot g^{-1}$]
$J_{m,O2}$	Mass-specific O_2 flux [$nmol \cdot s^{-1} \cdot g^{-1}$]
$J_{mte,O2}$	Mitochondria-specific O_2 flux [$nmol \cdot s^{-1} \cdot x_{mte}^{-1}$]
mte	Amount of mitochondrial elements [x_{mte}]
m_{thom}	Mass of tissue in the homogenate [g]
Y_{mt}	Yield of isolated mitochondria



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1056 **Fig. 8. Normalization of volume-specific flux of isolated mitochondria and tissue**
1057 **homogenate. A:** Mitochondrial yield, Y_{mt}, in preparation of isolated mitochondria. $v_{thom,1}$
1058 and $v_{stock,1}$ are the volumes transferred from the total volume, V_{thom} and V_{stock}, respectively.
1059 $mte_{thom,1}$ is the amount of mitochondrial elements in volume $v_{thom,1}$ used for isolation. **B:**
1060 In respirometry with homogenate, $v_{thom,1}$ is transferred directly into the respirometer
1061 chamber. See **Table 6** for further explanation of symbols.

1062

1063

1064

Table 7. Some useful abbreviations of various sample types, *X*.

Identity of sample	*X*
Mitochondrial preparations	mtprep
Isolated mitochondria	imt
Tissue homogenate	thom
Permeabilized tissue	pti
Permeabilized fibres	pfi
Permeabilized cells	pce
Cells	ce

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·mL^{-1}], where N_X is the number of cells or organisms in the chamber (**Table 6**).

Flow per sample entity, $I_{X,O2}$: 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, $I_{cell,O2}$ (**Table 6**). O_2 flow can be calculated from volume-specific O_2 flux, $J_{V,O2}$ [nmol·s^{-1}·L^{-1}] (per V of the measurement chamber [L]), divided by the number concentration of cells, $C_{Nce}=N_{ce}/V$ [cell·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).

The complexity changes when the sample is a whole organism studied as an experimental model. The well-established scaling law in respiratory physiology reveals a strong interaction of O_2 consumption and individual body mass of an organism, since *basal* metabolic rate (flow) does not increase linearly with body mass, whereas *maximum* mass-specific O_2 flux, $\dot{V}_{O2max}$ or

$\dot{V}_{O2peak}$, 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. $\dot{V}_{O2peak}$ of human endurance athletes is 60 to 80 mL $O_2 \cdot min^{-1} \cdot kg^{-1}$ body mass, converted to $J_{m,O2peak}$ of 45 to 60 $nmol \cdot s^{-1} \cdot g^{-1}$ (Gnaiger 2014; **Table 8**).

4.3. Normalization for mitochondrial content

Normalization is a problematic subject and it is essential 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 tissue, then normalization can be successful, using tissue mass or protein content, for example. 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. However, one cannot assume that quantitative changes in various markers such as mitochondrial proteins necessarily occur in parallel with one another. It is important to first establish that the marker chosen is not selectively altered by the performed treatment. In conclusion, the normalization must reflect the question under investigation to reach a satisfying answer. On the other hand, the goal of comparing results across projects and institutions requires some standardization on normalization for entry into a databank.

Mitochondrial concentration, C_{mte}, and mitochondrial markers: It is important that mitochondrial content in the tissue and the measurement chamber be quantified, as a physiological output and result of mitochondrial biogenesis and degradation, and as a quantity for normalization in functional analyses. Mitochondrial organelles comprise a cellular reticulum that is in a continual flux of fusion and fission. Hence the definition of an "amount" of mitochondria is often misconceived: mitochondria cannot be counted as a number of occurring elements. Therefore, quantification of the "amount" of mitochondria depends on measurement of chosen mitochondrial markers. ‘Mitochondria are the structural and functional elemental units of cell respiration’ (Gnaiger 2014). The quantity 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, particularly in mitochondrial dysfunction, some markers can vary while other markers are unchanged. (*1*) Mitochondrial volume or membrane area are structural markers, whereas mitochondrial protein mass is frequently used as a marker 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, cardiolipin, TOM20. (*3*) Extending the measurement of mitochondrial marker enzyme activity to mitochondrial pathway capacity, measured as 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, $C_{mte}=D_{mte}\cdot C_{mX}$, or mitochondrial content multiplied by sample number concentration, $C_{mte}=mte_X\cdot C_{NX}$ (**Table 6**).

Mitochondria-specific flux, $J_{mte,O2}$: Volume-specific metabolic O_2 flux depends on: (*1*) the sample concentration in the volume of the instrument chamber, C_{mX}, or C_{NX}; (*2*) the mitochondrial density in the sample, D_{mte}=mte/m_X or mte_X=mte/N_X; and (*3*) the specific mitochondrial activity or performance per elemental mitochondrial unit, $J_{mte,O2}=J_{V,O2}/C_{mte}$ (**Table 6**). Obviously, the numerical results for $J_{mte,O2}$ vary according to the type of mitochondrial marker chosen for measurement of mte and C_{mte}=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 inaccurate and noisy if

normalized for a biased and noisy measurement of a mitochondrial marker. This problem is acute in mitochondrial respiration because the denominators used (the mitochondrial marker) are often very small moieties whose accurate and precise determination is difficult. This problem can be 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*s (**Fig. 7**). *FCR*s are independent of any *externally* measured markers and, therefore, are statistically very robust. *FCR*s indicate qualitative changes of mitochondrial respiratory control, with highest quantitative resolution, separating the effect of mitochondrial density or concentration on $J_{mX,O2}$ or $I_{X,O2}$ from that of function per elemental mitochondrial marker, $J_{mte,O2}$ (Pesta *et al.* 2011; Gnaiger 2014). (*2*) If mitochondrial quality does not change and only the amount of mitochondria, defined by the chosen mitochondrial marker, 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 and precision of measurement of the mitochondrial marker. (*3*) If mitochondrial flux control ratios change, then there may not be any best mitochondrial marker. In general, measurement of multiple mitochondrial markers enables a comparison and evaluation of normalization for a variety of mitochondrial markers.

4.4. Conversion: units and normalization

Many different units have been used to report the rate of oxygen consumption, OCR (**Table 8**). *SI* base units provide the common reference for introducing the theoretical principles (**Fig. 7**), and are used with appropriately chosen *SI* prefixes to express numerical data in the most practical format, with an effort towards unification within specific areas of application (**Table 9**). For studies of cells, we recommend that respiration be expressed, as far as possible, as (*1*) O_2 flux normalized for a mitochondrial marker, for separation of the effects of mitochondrial quality and content on cell respiration (this includes *FCR*s as a normalization for

a functional mitochondrial marker); (*2*) O_2 flux in units of cell volume or mass, for comparison of respiration of cells with different cell size (Renner *et al.* 2003) and with studies on tissue preparations, and (*3*) O_2 flow in units of attomole (10^{-18} mol) of O_2 consumed by each cell in a second [amol·s^{-1}·cell^{-1}], numerically equivalent to [pmol·s^{-1}·10^{-6} cells]. This convention allows information to be easily used when designing experiments in which oxygen consumption must be considered. For example, to estimate the volume-specific O_2 flux in an instrument 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·s^{-1}·cell^{-1} and a cell density of 10^9 cells·L^{-1} (10^6 cells·mL^{-1}), the volume-specific O_2 flux is 100 nmol·s^{-1}·L^{-1} (100 pmol·s^{-1}·mL^{-1}).

Table 8. 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	Note
ng.atom O·s^{-1}	(2 e)		0.5	nmol O_2·s^{-1}	
ng.atom O·min^{-1}	(2 e)		8.33	pmol O_2·s^{-1}	
natom O·min^{-1}	(2 e)		8.33	pmol O_2·s^{-1}	
nmol O_2·min^{-1}	(4 e)		16.67	pmol O_2·s^{-1}	
nmol O_2·h^{-1}	(4 e)		0.2778	pmol O_2·s^{-1}	
mL O_2·min^{-1} at STPD[a]			0.744	µmol O_2·s^{-1}	1
W = J/s at -470 kJ/mol O_2			-2.128	µmol O_2·s^{-1}	
mA = mC·s^{-1}	(z_{H+}=1)		10.36	nmol H^+·s^{-1}	2
mA = mC·s^{-1}	(z_{O2}=4)		2.59	nmol O_2·s^{-1}	2
nmol H^+·s^{-1}	(z_{H+}=1)		0.09649	mA	3
nmol O_2·s^{-1}	(z_{O2}=4)		0.38594	mA	3

1189 1 At standard temperature and pressure dry (STPD: 0 °C=273.15 K and 1
1190 atm=101.325 kPa=760 mmHg), the molar volume of an ideal gas, V_m, and $V_{m,O2}$
1191 is 22.414 and 22.392 L.mol^{-1} respectively. Rounded to three decimal places, both
1192 values yield the conversion factor of 0.744. For comparison at NTPD (20 °C),
1193 $V_{m,O2}$ is 24.038 L.mol^{-1}. Note that the *SI* standard pressure is 100 kPa.
1194 2 The multiplication factor is $10^6/(z_B \cdot F)$.
1195 3 The multiplication factor is $z_B \cdot F/10^6$.
1196

1197 Although volume is expressed as m^3 using the *SI* base unit, the litre [dm^3] is the basic unit
1198 of volume for concentration and is used for most solution chemical kinetics. If one multiplies
1199 $I_{cell,O2}$ by C_{Ncell}, then the result will not only be the amount of O_2 [mol] consumed per time [s^{-1}]
1200 in one litre [L^{-1}], but also the change in the concentration of oxygen per second (for any volume
1201 of an ideally closed system). This is ideal for kinetic modeling as it blends with chemical rate
1202 equations where concentrations are typically expressed in mol·L^{-1} (Wagner *et al.* 2011). In
1203 studies of multinuclear cells, such as differentiated skeletal muscle cells, it is easy to determine
1204 the number of nuclei but not the total number of cells. A generalized concept, therefore, is
1205 obtained by substituting cells by nuclei as the sample entity. This does not hold, however, for
1206 enucleated platelets.
1207

1208 *4.5. Conversion: oxygen, proton and ATP flux*

1209 $J_{O2,k}$ is coupled in mitochondrial steady states to proton cycling, $J_{\infty H+} = J_{H+,out} = J_{H+,in}$ (**Fig.**
1210 **2**). $J_{H+,out/n}$ and $J_{H+,in/n}$ [nmol·s^{-1}·L^{-1}] are converted into electrical units, $J_{H+,out/e}$
1211 [mC·s^{-1}·L^{-1}=mA·L^{-1}] = $J_{H+,out/n}$ [nmol·s^{-1}·L^{-1}]·F [C·mol^{-1}]·10^{-6} (**Table 4**). At a $J_{H+,out}/J_{O2,k}$ ratio
1212 or H^+_{out}/O_2 of 20 (H^+_{out}/O=10), a volume-specific O_2 flux of 100 nmol·s^{-1}·L^{-1} would correspond
1213 to a proton flux of 2,000 nmol H^+_{out}·s^{-1}·L^{-1} or volume-specific current of 193 mA·L^{-1}.

1214 $$J_{V,H+out/e}\ [\text{mA}\cdot\text{L}^{-1}] = J_{V,H+out/n} \cdot F \cdot 10^{-6}\ [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}\cdot\text{mC}\cdot\text{nmol}^{-1}] \qquad \text{(Eq. 3.1)}$$

$$J_{V,H+out/e}\ [mA\cdot L^{-1}] = J_{V,O2}\cdot(H^{+}_{out}/O_2)\cdot F\cdot 10^{-6}\ [mC\cdot s^{-1}\cdot L^{-1}=mA\cdot L^{-1}] \quad \text{(Eq. 3.2)}$$

Table 9. Conversion for units with preservation of numerical values.

Name	Frequently used unit	Equivalent unit	Note
Volume-specific flux, $J_{V,O2}$	$pmol\cdot s^{-1}\cdot mL^{-1}$	$nmol\cdot s^{-1}\cdot L^{-1}$	1
	$mmol\cdot s^{-1}\cdot L^{-1}$	$mol\cdot s^{-1}\cdot m^{-3}$	
Cell-specific flow, I_{O2}	$pmol\cdot s^{-1}\cdot 10^{-6}$ cells	$amol\cdot s^{-1}\cdot cell^{-1}$	2
	$pmol\cdot s^{-1}\cdot 10^{-9}$ cells	$zmol\cdot s^{-1}\cdot cell^{-1}$	3
Cell number concentration, C_{Nce}	10^6 cells$\cdot mL^{-1}$	10^9 cells$\cdot L^{-1}$	
Mitochondrial protein concentration, C_{mte}	$0.1\ mg\cdot mL^{-1}$	$0.1\ g\cdot L^{-1}$	
Mass-specific flux, $J_{m,O2}$	$pmol\cdot s^{-1}\cdot mg^{-1}$	$nmol\cdot s^{-1}\cdot g^{-1}$	4
Catabolic power, $P_{k,O2}$	$\mu W\cdot 10^{-6}$ cells	$pW\cdot cell^{-1}$	1
Volume	1,000 L	m^3 (1,000 kg)	
	L	dm^3 (kg)	
	mL	cm^3 (g)	
	µL	mm^3 (mg)	
	fL	μm^3 (pg)	
Amount of substance concentration	M = $mol\cdot L^{-1}$	$mol\cdot dm^{-3}$	

1 pmol: picomole = 10^{-12} mol
2 amol: attomole = 10^{-18} mol
3 zmol: zeptomole = 10^{-21} mol
4 nmol: nanomole = 10^{-9} mol

ETS capacity in various human cell types including HEK 293, primary HUVEC and fibroblasts ranges from 50 to 180 $amol\cdot s^{-1}\cdot cell^{-1}$, measured in intact cells in the noncoupled state (see Gnaiger 2014). At 100 $amol\cdot s^{-1}\cdot cell^{-1}$ corrected for ROX (corresponding to a catabolic power of -48 $pW\cdot cell^{-1}$), the current across the mt-membranes, I_e, approximates 193 $pA\cdot cell^{-1}$ 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. Modelling approaches illustrate the link between proton motive force and currents (Willis *et al.* 2016).For NADH- and succinate-linked respiration, the mechanistic P»/O_2 ratio (referring to the full 4 electron reduction of O_2) is calculated at 20/3.7 and 12/3.7, respectively (Eq. 4) equal to 5.4 and 3.3. The classical P»/O ratios (referring to the 2 electron reduction of 0.5 O_2) 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),

$$P\text{»}/O_2 = (H^+_{out}/O_2)/(H^+_{in}/P\text{»}) \quad \text{(Eq. 4)}$$

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

$$J_{V,P\text{»}}\ [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}] = J_{V,O2}\cdot(H^+_{out}/O_2)/(H^+_{in}/P\text{»}) \quad \text{(Eq. 5.1)}$$

$$J_{V,P\text{»}}\ [\text{nmol}\cdot\text{s}^{-1}\cdot\text{L}^{-1}] = J_{V,O2}\cdot(P\text{»}/O_2) \quad \text{(Eq. 5.2)}$$

We consider isolated mitochondria as powerhouses and proton pumps as molecular machines to relate experimental results to energy metabolism of the intact cell. The cellular P»/O_2 based on oxidation of glycogen is increased by the glycolytic (fermentative) substrate-level phosphorylation of 3 P»/Glyc, *i.e.*, 0.5 mol P» for each mol O_2 consumed in the complete oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial P»/O_2 ratio of 5.4 yields a bioenergetic cell physiological P»/O_2 ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either by the malate-aspartate shuttle or by the glycerophosphate shuttle resulting in different theoretical yield of ATP generated by mitochondria, the energetic cost of which potentially must be taken into account. Considering also substrate-level phosphorylation in the TCA cycle, this high P»/O_2 ratio not only reflects proton translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger 1993a).

5. Conclusions

MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory defects linked to genetic variation, age-related health risks, sex-specific mitochondrial performance, lifestyle with its effects on degenerative diseases, and thermal and chemical environment. The present recommendations on coupling control states and rates, linked to the concept of the protonmotive force (Part 1) will be extended in a series of reports on pathway control of

mitochondrial respiration, respiratory states in intact cells, and harmonization of experimental procedures.

Box 5: Mitochondrial and cell respiration

Mitochondrial and cell respiration is the process of highly exothermic energy transformation in which scalar redox reactions are coupled to vectorial ion translocation across a semipermeable membrane, which separates the small volume of a bacterial cell or mitochondrion from the larger volume of its surroundings. The electrochemical exergy can be partially conserved in the phosphorylation of ADP to ATP or in ion pumping, or dissipated in an electrochemical short-circuit. Respiration is thus clearly distinguished from fermentation as the counterpart of cellular core energy metabolism. Respiration is separated in mitochondrial preparations from the partial contribution of fermentative pathways of the intact cell. According to this definition, residual oxygen consumption, as measured after inhibition of the mitochondrial electron transfer system, does not belong to the class of catabolic reactions and is, therefore, subtracted from total oxygen consumption to obtain baseline-corrected respiration.

The optimal choice for expressing mitochondrial and cell respiration (**Box 5**) as 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, respiratory reference state) is guided by the scientific question. Interpretation of the obtained data depends critically on appropriate normalization, and therefore reporting rates merely as $nmol \cdot s^{-1}$ is discouraged, since it restricts the analysis to intra-experimental comparison of relative (qualitative) differences. Expressing O_2 consumption per cell may not be possible when dealing with tissues. For studies with mitochondrial preparations, 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 g cell or tissue protein, or per cell or tissue 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 multiple normalizations, maximum 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, such as citrate synthase activity as an enzymatic matrix marker, 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.

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