Mitochondrial respiratory states and rates: Building blocks of mitochondrial physiology Part 1

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Mitochondrial respiratory states: Building blocks of mitochondrial physiology Part 1

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Abstract As the knowledge base and importance of mitochondrial physiology to human health expands, the necessity for harmonizing the terminology concerning mitochondrial respiratory states and rates has become increasingly apparent. The chemiosmotic theory establishes the mechanism of energy transformation and coupling in oxidative phosphorylation. The unifying concept of the protonmotive force provides the framework for developing a consistent theoretical foundation of mitochondrial physiology and bioenergetics. We follow IUPAC guidelines on terminology in physical chemistry, extended by considerations on open systems and thermodynamics of irreversible processes. The concept-driven constructive terminology incorporates the meaning of each quantity and aligns concepts and symbols to the nomenclature of classical bioenergetics. We endeavour to provide a balanced view on mitochondrial respiratory control and a critical discussion on reporting data of mitochondrial respiration in terms of metabolic flows and fluxes. Uniform standards for evaluation of respiratory states and rates will ultimately contribute to reproducibility between laboratories and thus support the development of databases of mitochondrial respiratory function in species, tissues, and cells. Clarity of concept and consistency of nomenclature facilitate effective transdisciplinary communication, education, and ultimately further discovery.

Keywords: Mitochondrial respiratory control, coupling control, mitochondrial preparations, protonmotive force, uncoupling, oxidative phosphorylation, OXPHOS, efficiency, electron transfer, ET; electron transfer system, ETS; proton leak, LEAK, residual oxygen consumption, ROX, State 2, State 3, State 4, normalization, flow, flux, O2

Executive summary

In view of the broad implications for health care, mitochondrial researchers face an increasing responsibility to disseminate their fundamental knowledge and novel discoveries to a wide range of stakeholders and scientists beyond the group of specialists. This requires implementation of a commonly accepted terminology within the discipline and standardization in the translational context. Authors, reviewers, journal editors, and lecturers are challenged to collaborate with the aim to harmonize the nomenclature in the growing field of mitochondrial physiology and bioenergetics, from evolutionary biology and comparative physiology to mitochondrial medicine. In the present communication we focus on the following concepts in mitochondrial physiology:

1. Aerobic respiration depends on the coupling of phosphorylation (ADP → ATP) to O2 flux in catabolic reactions. Coupling in oxidative phosphorylation is mediated by the translocation of protons across the inner mitochondrial membrane through proton pumps generating or utilizing the protonmotive force, that is maintained between the mitochondrial matrix and intermembrane compartment or outer mitochondrial space. Compartmental coupling distinguishes this vectorial component of oxidative phosphorylation from glycolytic fermentation as the counterpart of cellular core energy metabolism (Figure 1). Cell respiration is distinguished from fermentation: (1) Electron acceptors are supplied by external respiration for the maintenance of redox balance, whereas fermentation is characterized by an internal electron acceptor produced in intermediary metabolism. In aerobic cell respiration, redox balance is maintained by O2 as the electron acceptor. (2) Compartmental coupling in vectorial oxidative phosphorylation contrasts to exclusively scalar substrate-level phosphorylation in fermentation.

2. When measuring mitochondrial metabolism, the contribution of fermentation and other cytosolic interactions must be excluded from analysis by disrupting the barrier function of the plasma membrane. Selective removal or permeabilization of the plasma membrane yields mitochondrial preparations—including isolated mitochondria, tissue and cellular preparations—with structural and functional integrity. Subsequently, extra-mitochondrial concentrations of fuel substrates, ADP, ATP, inorganic phosphate, and cations including H+ can be controlled to determine mitochondrial function under a set of conditions defined as coupling control states. We strive to incorporate an easily recognized and understood, concept-driven terminology of bioenergetics with explicit terms and symbols that define the nature of respiratory states.

3. Mitochondrial coupling states are defined according to the control of respiratory oxygen flux by the protonmotive force. Capacities of oxidative phosphorylation and electron transfer are
measured at kinetically saturating concentrations of fuel substrates, ADP and inorganic phosphate, and O₂, or at optimal uncoupler concentrations, respectively, in the absence of Complex IV inhibitors such as NO, CO, or H₂S. Respiratory capacity is a measure of the upper bound of the rate of respiration; it depends on the substrate type undergoing oxidation, and provides reference values for the diagnosis of health and disease, and for evaluation of the effects of Evolutionary background, Age, Gender and sex, Lifestyle and Environment.

Figure 1. Internal and external respiration
Mitochondrial respiration is the oxidation of fuel substrates (electron donors) and reduction of O₂ catalysed by the electron transfer system, ETS: (mt) mitochondrial catabolic respiration; (ce) total cellular O₂ consumption; and (ext) external respiration. All chemical reactions, r, that consume O₂ in the cells of an organism, contribute to cell respiration, \( J_{O_2} \). In addition to mitochondrial catabolic respiration, O₂ is consumed by:

1. Mitochondrial residual O₂ consumption, \( R_{O_2} \).
2. Non-mitochondrial O₂ consumption by catabolic reactions, particularly peroxisomal oxidases and microsomal cytochrome P450 systems.
3. Non-mitochondrial \( R_{O_2} \) by reactions unrelated to catabolism.
4. Extracellular \( R_{O_2} \).
5. Aerobic microbial respiration. Bars are not at a quantitative scale.

(mt) **Mitochondrial catabolic respiration**, \( J_{O_2} \), is the O₂ consumption by the mitochondrial ETS excluding \( R_{O_2} \).

(ce) **Cell respiration**, \( J_{O_2} \), takes into account internal O₂-consuming reactions, r, including catabolic respiration and \( R_{O_2} \). Catabolic cell respiration is the O₂ consumption associated with catabolic pathways in the cell, including mitochondrial catabolism in addition to peroxisomal and microsomal oxidation reactions (4).

(ext) **External respiration** balances internal respiration at steady-state, including extracellular \( R_{O_2} \) and aerobic respiration by the microbiome (6). O₂ is transported from the environment across the respiratory cascade, i.e., circulation between tissues and diffusion across cell membranes, to the intracellular compartment. The respiratory quotient, \( RQ \), is the molar CO₂/O₂ exchange ratio; when combined with the respiratory nitrogen quotient, \( N/O_2 \) (mol N given off per mol O₂ consumed), the \( RQ \) reflects the proportion of carbohydrate, lipid and protein utilized in cell respiration during aerobically balanced steady-states. Bicarbonate and CO₂ are transported in reverse to the extracellular milieu and the organismic environment. Hemoglobin provides the molecular paradigm for the combination of O₂ and CO₂ exchange, as do lungs and gills on the morphological level.

4. Incomplete tightness of coupling, i.e., some degree of uncoupling relative to the substrate-dependent stoichiometry, is a characteristic of energy-transformations across membranes. Uncoupling is caused by a variety of physiological, pathological, toxicological, pharmacological and environmental conditions that exert an influence not only on the proton leak and cation cycling, but also on proton slip within the proton pumps and the structural integrity of the mitochondria. A more loosely coupled state is induced by stimulation of mitochondrial superoxide formation and the bypass of proton pumps. In addition, the use of protonophores represents an experimental uncoupling intervention to assess the transition from a well-coupled to a noncoupled state of mitochondrial respiration.

5. Respiratory oxygen consumption rates have to be carefully normalized to enable meta-analytic studies beyond the question of a particular experiment. Therefore, all raw data on rates and variables for normalization should be published in an open access data repository. Normalization of rates for: (1) the number of objects (cells, organisms); (2) the volume or
mass of the experimental sample; and (3) the concentration of mitochondrial markers in the experimental chamber are sample-specific normalizations, which are distinguished from system-specific normalization for the volume of the chamber (the measuring system).

6. The consistent use of terms and symbols will facilitate transdisciplinary communication and support the further development of a collaborative database on bioenergetics and mitochondrial physiology. The present considerations are focused on studies with mitochondrial preparations. These 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 1: In brief – Mitochondria and Bioblasts**

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

Mitochondria are oxygen-consuming electrochemical generators that evolved from the endosymbiotic alphaproteobacteria which integrated into a host cell related to Asgard Archaea (Margulis 1970; Lane 2005; Roger et al. 2017). They were described by Richard Altman (1894) as ‘bioblasts’, which include not only the mitochondria as presently defined, but also symbiotic and free-living bacteria. The word ‘mitochondria’ (Greek mitos: thread; chondros: granule) was introduced by Carl Benda (1898).

Contrary to current textbook dogma, mitochondria form dynamic networks within eukaryotic cells. Mitochondrial movement is supported by microtubules and morphology can change in response to energy requirements of the cell via processes known as fusion and fission; these interactions allow mitochondria to communicate within a network (Chan 2006). Mitochondria can even traverse cell boundaries in a process known as horizontal mitochondrial transfer (Torralba et al. 2016). Another defining characteristic of mitochondria is the double membrane. The mitochondrial inner membrane (mtIM) forms dynamic tubular to disk-shaped cristae that separate the mitochondrial matrix, i.e., the negatively charged internal mitochondrial compartment, from the intermembrane space; the latter being enclosed by the mitochondrial outer membrane (mtOM) and positively charged with respect to the matrix. The mtIM contains the non-bilayer phospholipid cardiolipin, which is not present in any other eukaryotic cellular membrane. Cardiolipin has many regulatory functions (Oemer et al. 2018); in particular, it stabilizes and promotes the formation of respiratory supercomplexes (SC $I_{IIIIV}$), which are supramolecular assemblies based upon specific and dynamic interactions between individual respiratory complexes (Greggio et al. 2017; Lenaz et al. 2017). The fluidity of the mitochondrial membrane is plastic and exerts an influence on the functional properties of proteins incorporated in membranes (Waczulikova et al. 2007). Intracellular stress factors may cause shrinking or swelling of the mitochondrial matrix, that can ultimately result in permeability transition.

Mitochondria are the structural and functional elementary components of cell respiration. Mitochondrial respiration is the reduction of molecular oxygen by electron transfer coupled to electrochemical proton translocation across the mtIM. In the process of oxidative phosphorylation (OXPHOS), the catabolic reaction of oxygen consumption is electrochemically coupled to the transformation of energy in the form of adenosine triphosphate (ATP; Mitchell 1961, 2011). Mitochondria are the powerhouses of the cell which contain the machinery of the OXPHOS-pathways, including transmembrane respiratory complexes (proton pumps with FMN, Fe-S and cytochrome $b, c$, $aa_3$ redox systems); alternative dehydrogenases and oxidases; the coenzyme ubiquinone (Q); F-ATPase or ATP synthase; the enzymes of the tricarboxylic acid cycle (TCA), fatty acid and amino acid oxidation; transporters of ions, metabolites and co-factors; iron/sulphur cluster synthesis; and mitochondrial kinases related to catabolic pathways. The mitochondrial proteome comprises over 1,200 proteins (Calvo et al. 2015; 2017), mostly encoded by nuclear DNA (nDNA), with a variety of functions, many of which are relatively well known (e.g., proteins regulating mitochondrial biogenesis or apoptosis), while others are still under investigation, or need to be identified (e.g., permeability transition pore, alanine transporter). Only recently has it been possible to use the mammalian mitochondrial proteome to discover and characterize the genetic basis of mitochondrial diseases (Williams et al. 2016; Palmfeldt and Bross 2017).

Numerous cellular processes are orchestrated by a constant crosstalk between mitochondria and other cellular components. For example, the crosstalk between mitochondria and the endoplasmic
Mitochondria are the powerhouses of the cell with numerous physiological, molecular, and genetic functions (Box 1). Every study of mitochondrial health and disease faces Evolution, Age, Gender and sex, Lifestyle, and Environment (MitoEAGLE) as essential background conditions intrinsic to the individual person or cohort, species, tissue and to some extent even cell line. As a large and coordinated group of laboratories and researchers, the mission of the global MitoEAGLE Network is to generate the necessary scale, type, and quality of consistent data sets and conditions to address this intrinsic complexity. Harmonization of experimental protocols and implementation of a quality control
and data management system are required to interrelate results gathered across a spectrum of studies and to generate a rigorously monitored database focused on mitochondrial respiratory function. In this way, researchers from a variety of disciplines can compare their findings using clearly defined and accepted international standards.

With an emphasis on quality of research, published data can be useful far beyond the specific question of a particular experiment. For example, collaborative data sets support the development of open-access databases such as those for National Institutes of Health sponsored research in genetics, proteomics, and metabolomics. Indeed, enabling meta-analysis is the most economic way of providing robust answers to biological questions (Cooper et al. 2009). However, the reproducibility of quantitative results and databases depend on accurate measurements under strictly-defined conditions. Likewise, meaningful interpretation and comparability of experimental outcomes requires standardisation of protocols between research groups at different institutes. In addition to quality control, a conceptual framework is also required to standardise and homogenise terminology and methodology. Vague or ambiguous jargon can lead to confusion and may relegate valuable signals to wasteful noise. For this reason, measured values must be expressed in standard units for each parameter used to define mitochondrial respiratory function. A consensus on fundamental nomenclature and conceptual coherence, however, are missing in the expanding field of mitochondrial physiology. To fill this gap, the present communication provides an in-depth review on harmonization of nomenclature and definition of technical terms, which are essential to improve the awareness of the intricate meaning of current and past scientific vocabulary. This is important for documentation and integration into databases in general, and quantitative modelling in particular (Beard 2005).

In this review, we focus on coupling states and fluxes through metabolic pathways of aerobic energy transformation in mitochondrial preparations as a first step in the attempt to generate a conceptually-oriented nomenclature in bioenergetics and mitochondrial physiology. Respiratory control by fuel substrates and specific inhibitors of respiratory enzymes, coupling states of intact cells, and respiratory flux control ratios will be reviewed in subsequent communications, prepared in the frame of COST Action MitoEAGLE open to global bottom-up input.

### 2. Coupling states and rates in mitochondrial preparations

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

#### 2.1. Cellular and mitochondrial respiration

##### 2.1.1. Aerobic and anaerobic catabolism and ATP turnover: In respiration, electron transfer is coupled to the phosphorylation of ADP to ATP, with energy transformation mediated by the protonmotive force, pmf (Figure 2). Anabolic reactions are coupled to catabolism, both by ATP as the intermediary energy currency and by small organic precursor molecules as building blocks for biosynthesis. Glycolysis involves substrate-level phosphorylation of ADP to ATP in fermentation without utilization of O₂, studied mainly in intact cells and organisms. Many cellular fuel substrates are catabolized to acetyl-CoA or to glutamate, and further electron transfer reduces nicotinamide adenine dinucleotide to NADH or flavin adenine dinucleotide to FADH₂. Subsequent mitochondrial electron transfer to O₂ is coupled to proton translocation for the control of the protonmotive force and phosphorylation of ADP (Figure 2B and 2C). In contrast, extra-mitochondrial oxidation of fatty acids and amino acids proceeds partially in peroxisomes without coupling to ATP production: acyl-CoA oxidase catalyzes the oxidation of FADH₂ with electron transfer to O₂; amino acid oxidases oxidize flavin mononucleotide FMNH₂ or FADH₂ (Figure 2A).
Mitochondrial respiration is the oxidation of fuel substrates (electron donors) with electron transfer to $O_2$ as the electron acceptor. For explanation of symbols see also Figure 1.

(A) Respiration of intact cells: Extra-mitochondrial catabolism of macrofuels and uptake of small molecules by the cell provide the mitochondrial fuel substrates. Dashed arrows indicate the connection between the redox proton pumps (respiratory Complexes CI, CIII and CIV) and the transmembrane protonmotive force, pmf. Coenzyme Q (Q) and the cytochromes $b$, $c$, and $aa_3$ are redox systems of the mitochondrial inner membrane, mtIM. Glycerol-3-phosphate, Gp.
(B) Respiration in mitochondrial preparations: The mitochondrial electron transfer system (ETS) is (J) fuelled by diffusion and transport of substrates across the mtOM and mtIM, and in addition consists of the (2) matrix-ETS, and (3) membrane-ETS. Electron transfer converges at the N-junction, and from CI, CII and electron transferring flavoprotein complex (CETF) at the Q-junction. Unspecified arrows converging at the Q-junction indicate additional ET-sections with electron entry into Q through glycerophosphate dehydrogenase, dihydro-orotate dehydrogenase, proline dehydrogenase, choline dehydrogenase, and sulfide-ubiquinone oxidoreductase. The dotted arrow indicates the branched pathway of oxygen consumption by alternative quinol oxidase (AOX). ET-paths are coupled to the phosphorylation-pathway. The H⁺/O₂ ratio is the outward proton flux from the matrix space to the positively (pos) charged vesicular compartment, divided by catabolic O₂ flux in the NADH-pathway. The H⁺/Pi ratio is the inward proton flux from the inter-membrane space to the negatively (neg) charged matrix space, divided by the flux of phosphorylation of ADP to ATP. These stoichiometries are not fixed due to ion leaks and proton slip. Modified from Lemieux et al. (2017) and Rich (2013).

(C) OXPHOS coupling: O₂ flux through the catabolic ET-pathway, \( j_{\text{O}_2} \), is coupled by the H⁺ circuit to flux through the phosphorylation-pathway of ADP to ATP, \( j_{\text{Pi}} \).

(D) Chemiosmotic phosphorylation-pathway catalyzed by the proton pump F₁F₀-ATPase (F-ATPase, ATP synthase), adenine nucleotide translocase (ANT), and inorganic phosphate carrier (PiC). The H⁺/Pi stoichiometry is the sum of the coupling stoichiometry in the F-ATPase reaction (-2.7 H⁺/Pi from the positive intermembrane space, 2.7 H⁺/Pi to the matrix, i.e., the negative compartment) and the proton balance in the translocation of ADP³⁻, ATP⁴⁺ and Pi⁵⁻. Modified from Gnaiger (2014).

The plasma membrane separates the intracellular compartment including the cytosol, nucleus, and organelles from the extracellular environment. The plasma membrane consists of a lipid bilayer with embedded proteins and attached organic molecules that collectively control the selective permeability of ions, organic molecules, and particles across the cell boundary. The intact plasma membrane prevents the passage of many water-soluble mitochondrial substrates and inorganic ions—such as succinate, adenosine diphosphate (ADP) and inorganic phosphate (Pi), that must be precisely controlled at kinetically-saturating concentrations for the analysis of mitochondrial respiratory capacities. Respiratory capacities delineate, comparable to channel capacity in information theory (Schneider 2006), the upper bound of the rate of O₂ consumption measured in defined respiratory states. Despite the activity of solute carriers, e.g., SLC13A3 and SLC20A2, which transport specific metabolites across the plasma membrane of various cell types, the intact plasma membrane limits the scope of investigations into mitochondrial respiratory function in intact cells.

2.1.2. Specification of biochemical dose: Substrates, uncouplers, inhibitors, and other chemical reagents are titrated to analyse cellular and mitochondrial function. Nominal concentrations of these substances are usually reported as initial amount of substance concentration [mol·L⁻¹] in the incubation medium. When aiming at the measurement of kinetically saturated processes—such as OXPHOS-capacities, the concentrations for substrates can be chosen according to the apparent equilibrium constant, \( K_m \). In the case of hyperbolic kinetics, only 80% of maximum respiratory capacity is obtained at a substrate concentration of four times the \( K_m \), whereas substrate concentrations of 5, 9, 19 and 49 times the \( K_m \) are theoretically required for reaching 83%, 90%, 95% or 98% of the maximal rate (Gnaiger 2001). Other reagents are chosen to inhibit or alter a particular process. The amount of these chemicals in an experimental incubation is selected to maximize effect, avoiding unacceptable off-target consequences that would adversely affect the data being sought. Specifying the amount of substance in an incubation as nominal concentration in the aqueous incubation medium can be ambiguous (Doskey et al. 2015), particularly for cations (TPP⁺; fluorescent dyes such as safranine, TMRM; Chowdhury et al. 2015) and lipophilic substances (oligomycin, uncouplers, permeabilization agents; Doerrier et al. 2018), which accumulate in the mitochondrial matrix or in biological membranes, respectively. Generally, dose/exposure can be specified per unit of biological sample, i.e., (nominal moles of xenobiotic)/(number of cells) [mol·cell⁻¹] or, as appropriate, per mass of biological sample [mol·kg⁻¹]. This approach to specification of dose/exposure provides a scalable parameter that can be used to design experiments, help interpret a wide variety of experimental results, and provide absolute information that allows researchers worldwide to make the most use of published data (Doskey et al. 2015).
2.2. Mitochondrial preparations

Mitochondrial preparations are defined as either isolated mitochondria, or tissue and cellular preparations in which the barrier function of the plasma membrane is disrupted. Since this entails the loss of cell viability, mitochondrial preparations are not studied in vivo. In contrast to isolated mitochondria and tissue homogenate preparations, mitochondria in permeabilized tissues and cells are in situ relative to the plasma membrane. When studying mitochondrial preparations, substrate-uncoupler-inhibitor-titration (SUIT) protocols are used to establish respiratory coupling control states (CCS) and pathway control states (PCS) that provide reference values for various output variables (Table 1). Physiological conditions in vivo deviate from these experimentally obtained states; this is because kinetically-saturating concentrations, e.g., of ADP, oxygen (O₂; dioxygen) or fuel substrates, may not apply to physiological intracellular conditions. Further information is obtained in studies of kinetic responses to variations in fuel substrate concentrations, [ADP], or [O₂] in the range between kinetically-saturating concentrations and anoxia (Gnaiger 2001).

The cholesterol content of the plasma membrane is high compared to mitochondrial membranes (Korn 1969). Therefore, mild detergents—such as digitonin and saponin—can be applied to selectively permeabilize the plasma membrane via interaction with cholesterol; this allows free exchange of organic molecules and inorganic ions between the cytosol and the immediate cell environment, while maintaining the integrity and localization of organelles, cytoskeleton, and the nucleus. Application of permeabilization agents (mild detergents or toxins) leads to washout of cytosolic marker enzymes—such as lactate dehydrogenase—and results in the complete loss of cell viability (tested by nuclear staining using plasma membrane-impermeable dyes), while mitochondrial function remains intact (tested by cytochrome c stimulation of respiration). Digitonin concentrations have to be optimized according to cell type, particularly since mitochondria from cancer cells contain significantly higher contents of cholesterol in both membranes (Baggetto and Testa-Perussini, 1990). For example, a dose of digitonin of 8 fmol·cell⁻¹ (10 pg·cell⁻¹; 10 μg·10⁶ cells) is optimal for permeabilization of endothelial cells, and the concentration in the incubation medium has to be adjusted according to the cell density applied (Doerrier et al. 2018). Respiration of isolated mitochondria remains unaltered after the addition of low concentrations of digitonin or saponin. In addition to mechanical cell disruption during homogenization of tissue, permeabilization agents may be applied to ensure permeabilization of all cells in tissue homogenates.

Suspensions of cells permeabilized in the respiration chamber and crude tissue homogenates contain all components of the cell at highly dilute concentrations. All mitochondria are retained in chemically-permeabilized mitochondrial preparations and crude tissue homogenates. In the preparation of isolated mitochondria, however, the mitochondria are separated from other cell fractions and purified by differential centrifugation, entailing the loss of mitochondria at typical recoveries ranging from 30% to 80% of total mitochondrial content (Lai et al. 2018). Using Percoll or sucrose density gradients to maximize the purity of isolated mitochondria may compromise the mitochondrial yield or structural and functional integrity. Therefore, mitochondrial isolation protocols need to be optimized according to each study. The term mitochondrial preparation does neither include intact cells, nor submitochondrial particles and further fractionation of mitochondrial components.

2.3. Electron transfer pathways

Mitochondrial electron transfer (ET) pathways are fuelled by diffusion and transport of substrates across the mtOM and mtIM. In addition, the mitochondrial electron transfer system (ETS) consists of the matrix-ETS, and membrane-ETS (Figure 2B). Upstream sections of ET-pathways converge at the NADH-junction (N-junction). NADH is mainly generated in the tricarboxylic acid (TCA) cycle and is oxidized by Complex I (CI), with further electron entry into the coenzyme Q-junction (Q-junction). Similarly, succinate is formed in the TCA cycle and oxidized by CI to fumarate. CI is part of both the TCA cycle and the ETS, and reduces FAD to FADH₂ with further reduction of ubiquinone to ubiquinol downstream of the TCA cycle in the Q-junction. Thus FADH₂ is not a substrate but is the product of CI, in contrast to erroneous metabolic maps shown in many publications. β-oxidation of fatty acids (FA) generates FADH₂ as the substrate of electron transferring flavoprotein complex (ETF).

Selected mitochondrial catabolic pathways, k, of electron transfer from the oxidation of fuel substrates to the reduction of O₂ are activated by depletion of endogenous substrates and addition of fuel
substrates to the mitochondrial respiration medium (Figure 2B). Substrate combinations and specific inhibitors of ET-pathway enzymes are used to obtain defined pathway control states in mitochondrial preparations (Gnaiger 2014).

2.4. Respiratory coupling control

2.4.1. Coupling: In mitochondrial electron transfer, vectorial transmembrane proton flux is coupled through the redox proton pumps CI, CIII and CIV to the catabolic flux of scalar reactions, collectively measured as O₂ flux, J_{\text{O₂}} (Figure 2). Thus mitochondria are elementary components of energy transformation. Energy is a conserved quantity and cannot be lost or produced in any internal process (First Law of thermodynamics). Open and closed systems can gain or lose energy only by external fluxes—by exchange with the environment. Therefore, energy can neither be produced by mitochondria, nor is there any internal process without energy conservation. Exergy or Gibbs energy (‘free energy’) is the part of energy that can potentially be transformed into work under conditions of constant temperature and pressure. Coupling is the interaction of an exergonic process (spontaneous, negative exergy change) with an endergonic process (positive exergy change) in energy transformations which conserve part of the exergy that would be irreversibly lost or dissipated in an uncoupled process.

Pathway control states (PCS) and coupling control states (CCS) are complementary, since mitochondrial preparations depend on (1) an exogenous supply of pathway-specific fuel substrates and oxygen, and (2) exogenous control of phosphorylation (Figure 2).

2.4.2. Phosphorylation, P», and P»/O₂ ratio: Phosphorylation in the context of OXPHOS is defined as phosphorylation of ADP by P, to form ATP. On the other hand, the term phosphorylation is used generally in many 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 (Figure 2). We propose the symbol P» for the endergonic (uphill) direction of phosphorylation ADP→ATP, and likewise the symbol P« for the corresponding exergonic (downhill) hydrolysis ATP→ADP. P» refers mainly to electrontransfer phosphorylation but may also involve substrate-level phosphorylation as part of the TCA cycle (succinyl-CoA ligase; phosphoglycerate kinase) and phosphorylation of ADP catalyzed by pyruvate kinase, and of GDP phosphorylated by phosphoenolpyruvate carboxykinase. Transphosphorylation is performed by adenylate kinase, creatine kinase (mtCK), hexokinase and nucleoside diphosphate kinase. In isolated mammalian mitochondria, ATP production catalyzed by adenylate kinase (2 ADP ↔ ATP + AMP) proceeds without fuel substrates in the presence of ADP (Komlodi and Tretter 2017). Kinase cycles are involved in intracellular energy transfer and signal transduction for regulation of energy flux.

The P»/O₂ ratio (P»/4 e⁻) is two times the ‘P’/O ratio (P»/2 e⁻) of classical bioenergetics. P»/O₂ is a generalized symbol, not specific for determination of P, consumption (P»/O₂ flux ratio), ADP depletion (ADP/O₂ flux ratio), or ATP production (ATP/O₂ flux ratio). The mechanistic P»/O₂ ratio—or P»/O₂ stoichiometry—is calculated from the proton-to-O₂ and proton-to-phosphorylation coupling stoichiometries (Figure 2B):

\[
P_\text{»}/O_2 = \frac{H_\text{pos}^{+}/O_2}{H_\text{neg}^{+}/P_\text{»}}
\]

(1)

The H"pos/O₂ coupling stoichiometry (referring to the full 4 electron reduction of O₂) depends on the relative involvement of the three coupling sites (respiratory Complexes CI, CIII and CIV) in the catabolic ET-pathway from reduced fuel substrates (electron donors) to the reduction of O₂ (electron acceptor). This varies with: (1) a bypass of CI by single or multiple electron input into the Q-junction; and (2) a bypass of CIV by involvement of alternative oxidases, AOX, which are not expressed in mammalian mitochondria.

The H"pos/O₂ coupling stoichiometry equals 12 in the ET-pathways involving CIII and CIV as proton pumps, increasing to 20 for the NADH-pathway through CI (Figure 2B), but a general consensus on H"pos/O₂ stoichiometries remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov 2015). The H"neg/P" coupling stoichiometry (3.7; Figure 2B) is the sum of 2.7 H"neg required by the F-ATPase of vertebrate and most invertebrate species (Watt et al. 2010) and the proton balance in the translocation of ADP, ATP and P, (Figure 2C). Taken together, the mechanistic P»/O₂ ratio is calculated at 5.4 and 3.3 for NADH- and succinate-linked respiration, respectively (Eq. 1). The corresponding classical P»/O ratios (referring to the 2 electron reduction of 0.5 O₂) are 2.7 and 1.6 (Watt et al. 2010), in agreement with the measured P»/O ratio for succinate of 1.58 ± 0.02 (Gnaiger et al. 2000).
An intact mitochondrial inner membrane, mtIM, is required for vectorial, compartmental coupling. ‘Acoupled’ respiration is the consequence of structural disruption with catalytic activity of non-compartmental mitochondrial fragments. Inducible uncoupling (e.g., by activation of UCP1) increases LEAK respiration; experimentally noncoupled respiration provides an estimate of ET-capacity obtained by titration of protonophores stimulating respiration to maximum O₂ flux. H⁺ leak-uncoupled, decoupled, and loosely coupled respiration are components of intrinsic uncoupling.

Pathological dysfunction may affect all types of uncoupling, including permeability transition, causing intrinsically dyscoupled respiration. Similarly, toxicological and environmental stress factors can cause extrinsically dyscoupled respiration. Reduced fuel substrates, red; oxidized products, ox.

2.4.3. Uncoupling: The effective P_/O₂ flux ratio (Y_{P_/O₂} = J_P_/J_{O₂}) is diminished relative to the mechanistic P_/O₂ ratio by intrinsic and extrinsic uncoupling or dyscoupling (Figure 3). Such generalized uncoupling is different from switching to mitochondrial pathways that involve fewer than three proton pumps (‘coupling sites’: Complexes CI, CIII and CIV), bypassing CI through multiple electron entries into the Q-junction, or CIII and CIV through AOX (Figure 2B). Reprogramming of mitochondrial pathways leading to different types of substrates being oxidized may be considered as a switch of gears (changing the stoichiometry by altering the substrate that is oxidized) rather than uncoupling (loosening the tightness of coupling relative to a fixed stoichiometry). In addition, Y_{P_/O₂} depends on several experimental conditions of flux control, increasing as a hyperbolic function of [ADP] to a maximum value (Gnaiger 2001).

Uncoupling of mitochondrial respiration is a general term comprising diverse mechanisms:

1. Proton leak across the mtIM from the pos– to the neg–compartment (H⁺ leak-uncoupled; Figure 3).
2. Cycling of other cations, strongly stimulated by permeability transition; comparable to the use of protonophores, cation cycling is experimentally induced by valinomycin in the presence of K⁺;
3. Decoupling by proton slip in the redox proton pumps when protons are effectively not pumped (CI, CIII and CIV) or are not driving phosphorylation (F-ATPase);
4. Loss of vesicular (compartmental) integrity when electron transfer is acoupled;
5. Electron leak in the loosely coupled univalent reduction of O₂ to superoxide (O₂⁻; superoxide anion radical).

Differences of terms—uncoupled vs. noncoupled—are easily overlooked, although they relate to different meanings of uncoupling (Figure 3 and Table 2).
2.5. Coupling states and respiratory rates

To extend the classical nomenclature on mitochondrial coupling states (Section 2.6) by a concept-driven terminology that explicitly incorporates information on the meaning of respiratory states, the terminology must be general and not restricted to any particular experimental protocol or mitochondrial preparation (Gnaiger 2009). Concept-driven nomenclature aims at mapping the meaning and concept behind the words and acronyms onto the forms of words and acronyms (Miller 1991). The focus of concept-driven nomenclature is primarily the conceptual why, along with clarification of the experimental how.

Table 1. Coupling states and residual oxygen consumption in mitochondrial preparations in relation to respiration- and phosphorylation-flux, \( J_{\text{KO}_2} \) and \( J_{\text{P}^+} \), and protonmotive force, pmf. Coupling states are established at kinetically-saturating concentrations of fuel substrates and \( O_2 \).

<table>
<thead>
<tr>
<th>State</th>
<th>( J_{\text{KO}_2} )</th>
<th>( J_{\text{P}^+} )</th>
<th>pmf</th>
<th>Inducing factors</th>
<th>Limiting factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEAK</td>
<td>( L ); low, cation leak-dependent respiration</td>
<td>0</td>
<td>max.</td>
<td>back-flux of cations including proton leak, proton slip</td>
<td>( J_{\text{P}^+} = 0 : (1) ) without ADP, ( L_N ); (2) max. ATP/ADP ratio, ( L_T ); or (3) inhibition of the phosphorylation-pathway, ( L_{\text{omyd}} )</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>( P ); high, ADP-stimulated respiration, OXPHOS-capacity</td>
<td>max.</td>
<td>high</td>
<td>kinetically-saturating [ADP] and [P]</td>
<td>( J_{\text{P}^+} ) by phosphorylation-pathway; or ( J_{\text{KO}_2} ) by ET-capacity</td>
</tr>
<tr>
<td>ET</td>
<td>( E ); max., noncoupled respiration, ET-capacity</td>
<td>0</td>
<td>low</td>
<td>optimal external uncoupler concentration for max. ( J_{O_2,E} )</td>
<td>( J_{\text{KO}_2} ) by ET-capacity</td>
</tr>
<tr>
<td>ROX</td>
<td>( Rox ); min., residual ( O_2 ) consumption</td>
<td>0</td>
<td>0</td>
<td>( J_{O_2,Rox} ) in non-ET-pathway oxidation reactions</td>
<td>inhibition of all ET-pathways; or absence of fuel substrates</td>
</tr>
</tbody>
</table>

To provide a diagnostic reference for respiratory capacities of core energy metabolism, the capacity of oxidative phosphorylation, OXPHOS, is measured at kinetically-saturating concentrations of ADP and P. The oxidative ET-capacity reveals the limitation of OXPHOS-capacity mediated by the phosphorylation-pathway. The ET- and phosphorylation-pathways comprise coupled segments of the OXPHOS-system. ET-capacity is measured as noncoupled respiration by application of external uncouplers. The contribution of intrinsically uncoupled \( O_2 \) consumption is studied by preventing the stimulation of phosphorylation either in the absence of ADP or by inhibition of the phosphorylation-pathway. The corresponding states are collectively classified as LEAK-states, when \( O_2 \) consumption compensates mainly for ion leaks, including the proton leak. Defined coupling states are induced by: (1) adding cation chelators such as EGTA, binding free \( Ca^{2+} \) and thus limiting cation cycling; (2) adding ADP and P; (3) inhibiting the phosphorylation-pathway; and (4) uncoupler titrations, while maintaining a defined ET-pathway state with constant fuel substrates and inhibitors of specific branches of the ET-pathway.

The three coupling states, ET, LEAK and OXPHOS, are shown schematically with the corresponding respiratory rates, abbreviated as \( E, L \) and \( P \), respectively (Figure 4). We distinguish metabolic pathways from metabolic states and the corresponding metabolic rates; for example: ET-pathways, ET-states, and ET-capacities, \( E \), respectively (Table 1). The protonmotive force is high in the OXPHOS-state when it drives phosphorylation, maximum in the LEAK-state of coupled mitochondria, driven by LEAK-respiration at a minimum back-flux of cations to the matrix side, and very low in the ET-state when uncouplers short-circuit the proton cycle (Table 1).
Respiratory states (ET, OXPHOS, LEAK; Table 1) and corresponding rates (E, P, L) are connected by the protonmotive force, pmf. (1) ET-capacity, E, is partitioned into (2) dissipative LEAK-respiration, L, when the Gibbs energy change of catabolic O$_2$ flux is irreversibly lost, (3) net OXPHOS-capacity, P-L, with partial conservation of the capacity to perform work, and (4) the excess capacity, E-P. Modified from Gnaiger (2014).

**Figure 4. Four-compartment model of oxidative phosphorylation**

**Figure 5. Respiratory coupling states**

(A) LEAK-state and rate, L: Oxidation only, since phosphorylation is arrested, $J_{P\rightarrow}$ = 0, and catabolic O$_2$ flux, $J_{K_{O_2}}$, is controlled mainly by the proton leak and slip, $J_{mH^+}$, at maximum protonmotive force (Figure 4). Extramitochondrial ATP may be hydrolyzed by extramitochondrial ATPases, $J_{P\leftarrow}$; then phosphorylation must be blocked.

(B) OXPHOS-state and rate, P: Oxidation coupled to phosphorylation, $J_{P\rightarrow}$, which is stimulated by kinetically-saturating [ADP] and [Pi], supported by a high protonmotive force. O$_2$ flux, $J_{K_{O_2}}$, is well-coupled at a P$_{\rightarrow}$/O$_2$ ratio of $J_{P\rightarrow}/J_{K_{O_2}}$. Extramitochondrial ATPases may recycle ATP, $J_{P\leftarrow}$.

(C) ET-state and rate, E: Oxidation only, since phosphorylation is zero, $J_{P\rightarrow}$ = 0, at optimum exogenous uncoupler concentration when noncoupled respiration, $J_{K_{O_2}}$, is maximum. The F-ATPase may hydrolyze extramitochondrial ATP.

2.5.1. **LEAK-state (Figure 5A):** The LEAK-state is defined as a state of mitochondrial respiration when O$_2$ flux mainly compensates for ion leaks in the absence of ATP synthesis, at kinetically-saturating concentrations of O$_2$, respiratory fuel substrates and P. LEAK-respiration is measured to obtain an estimate of intrinsic uncoupling without addition of an experimental uncoupler: (1) in the absence of adenylates, i.e., AMP, ADP and ATP; (2) after depletion of ADP at a maximum ATP/ADP ratio; or (3) after inhibition of the phosphorylation-pathway by inhibitors of F-ATPase—such as oligomycin, or of adenine nucleotide translocase—such as carboxyatractyloside. Adjustment of the nominal concentration of these inhibitors to the density of biological sample applied can minimize or avoid inhibitory side-effects exerted on ET-capacity or even some dyscoupling.
### Table 2. Terms on respiratory coupling and uncoupling.

<table>
<thead>
<tr>
<th>Term</th>
<th>$J_{1O_2}$</th>
<th>$P_{\text{ET}}/O_2$</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>acoupled</td>
<td>0</td>
<td></td>
<td>electron transfer in mitochondrial fragments without vectorial proton translocation (Figure 3)</td>
</tr>
<tr>
<td>proton leak-uncoupled</td>
<td>L</td>
<td>0</td>
<td>non-phosphorylating LEAK-respiration (Figure 5A)</td>
</tr>
<tr>
<td>decoupled</td>
<td>0</td>
<td></td>
<td>component of $L$, proton diffusion across the mtIM (Figure 3)</td>
</tr>
<tr>
<td>loosely coupled</td>
<td>0</td>
<td></td>
<td>component of $L$, proton slip (Figure 3)</td>
</tr>
<tr>
<td>dyscoupled</td>
<td>0</td>
<td></td>
<td>pathologically, toxicologically, environmentally increased uncoupling, mitochondrial dysfunction</td>
</tr>
<tr>
<td>inducibly uncoupled</td>
<td>0</td>
<td></td>
<td>by UCP1 or cation (e.g., $Ca^{2+}$) cycling (Figure 3)</td>
</tr>
<tr>
<td>noncoupled</td>
<td>$E$</td>
<td>0</td>
<td>ET-capacity, non-phosphorylating respiration stimulated to maximum flux at optimum exogenous protonophore concentration (Figure 5C)</td>
</tr>
<tr>
<td>well-coupled</td>
<td>$P$</td>
<td>high</td>
<td>OXPHOS-capacity, phosphorylating respiration with an intrinsic LEAK component (Figure 5B)</td>
</tr>
<tr>
<td>fully coupled</td>
<td>$P - L$</td>
<td>max.</td>
<td>OXPHOS-capacity corrected for LEAK-respiration (Figure 4)</td>
</tr>
</tbody>
</table>

- **Proton leak and uncoupled respiration**: The intrinsic proton leak is the *uncoupled* leak current of protons in which protons diffuse across the mtIM in the dissipative direction of the downhill protonmotive force without coupling to phosphorylation (Figure 5A). The proton leak flux depends non-linearly on the protonmotive force (Garlid *et al.* 1989; Divakaruni and Brand 2011), which is a temperature-dependent property of the mtIM and may be enhanced due to possible contaminations by free fatty acids. Inducible uncoupling mediated by uncoupling protein 1 (UCP1) is physiologically controlled, e.g., in brown adipose tissue. UCP1 is a member of the mitochondrial carrier family that is involved in the translocation of protons across the mtIM (Klingenberg 2017). Consequently, this short-circuit lowers the protonmotive force and stimulates electron transfer, respiration, and heat dissipation in the absence of phosphorylation of ADP.

- **Cation cycling**: There can be other cation contributors to leak current including calcium and probably magnesium. Calcium influx is balanced by mitochondrial $Na^+/Ca^{2+}$ or $H^+/Ca^{2+}$ exchange, which is balanced by $Na^+/H^+$ or $K^+/H^+$ exchanges. This is another effective uncoupling mechanism different from proton leak (Table 2).

- **Proton slip and decoupled respiration**: Proton slip is the *decoupled* process in which protons are only partially translocated by a redox proton pump of the ET-pathways and slip back to the original vesicular compartment. The proton leak is the dominant contributor to the overall leak current in mammalian mitochondria incubated under physiological conditions at 37 °C, whereas proton slip increases at lower experimental temperature (Canton *et al.* 1995). Proton slip can also happen in association with the F-ATPase, in which the proton slips downhill across the pump to the matrix without contributing to ATP synthesis. In each case, proton slip is a property of the proton pump and increases with the pump turnover rate.

- **Electron leak and loosely coupled respiration**: Superoxide production by the ETS leads to a bypass of redox proton pumps and correspondingly lower $P_{\text{ET}}/O_2$ ratio. This depends on the actual site of electron leak and the scavenging of hydrogen peroxide by cytochrome c, whereby electrons may re-enter the ETS with proton translocation by CIV.
- Loss of compartmental integrity and uncoupled respiration: Electron transfer and catabolic O\textsubscript{2} flux proceed without compartmental proton translocation in disrupted mitochondrial fragments. Such fragments are an artefact of mitochondrial isolation, and may not fully fuse to re-establish structurally intact mitochondria. Loss of mtIM integrity, therefore, is the cause of uncoupled respiration, which is a nonvectorial dissipative process without control by the protonmotive force.

- Dyscoupled respiration: Mitochondrial injuries may lead to dyscoupling as a pathological or toxicological cause of uncoupled respiration. Dyscoupling may involve any type of uncoupling mechanism, e.g., opening the permeability transition pore. Dyscoupled respiration is distinguished from the experimentally induced noncoupled respiration in the ET-state (Table 2).

2.5.2. OXPHOS-state (Figure 5B): The OXPHOS-state is defined as the respiratory state with kinetically-saturating concentrations of O\textsubscript{2}, respiratory and phosphorylation substrates, and absence of exogenous uncoupler, which provides an estimate of the maximal respiratory capacity in the OXPHOS-state for any given ET-pathway state. Respiratory capacities at kinetically-saturating substrate concentrations provide reference values or upper limits of performance, aiming at the generation of data sets for comparative purposes. Physiological activities and effects of substrate kinetics can be evaluated relative to the OXPHOS-capacity.

As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated mitochondria (Gnaiger 2001; Puchowicz et al. 2004); greater [ADP] is required, particularly in permeabilized muscle fibres and cardiomyocytes, to overcome limitations by intracellular diffusion and by the reduced conductance of the mtOM (Jephthima 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 addition, saturating ADP concentrations need to be evaluated under different experimental conditions such as temperature (Lemieux et al. 2017) and with different animal models (Blier and Guderley, 1993). In permeabilized muscle fibre bundles of high respiratory capacity, the apparent \( K_a \) for ADP increases up to 0.5 mM (Saks et al. 1998), consistent with experimental evidence that >90% saturation is reached only at >5 mM ADP (Pesta and Gnaiger 2012). Similar ADP concentrations are also required for accurate determination of OXPHOS-capacity in human clinical cancer samples and permeabilized cells (Klepinin et al. 2016; Koit et al. 2017). Whereas 2.5 to 5 mM ADP is sufficient to obtain the actual OXPHOS-capacity in many types of permeabilized tissue and cell preparations, experimental validation is required in each specific case.

2.5.3. Electron transfer-state (Figure 5C): O\textsubscript{2} flux determined in the ET-state yields an estimate of ET-capacity. The ET-state is defined as the noncoupled state with kinetically-saturating concentrations of O\textsubscript{2}, respiratory substrate and optimum exogenous uncoupler concentration for maximum O\textsubscript{2} flux. Uncouplers are weak lipid-soluble acids which function as protonophores. These disrupt the barrier function of the mtIM and thus short circuit the protonmotive system, functioning like a clutch in a mechanical system. As a consequence of the nearly collapsed protonmotive force, the driving force is insufficient for phosphorylation, and \( J_{\text{p}} = 0 \). The most frequently used uncouplers are carbonyl cyanide m-chlorophenyl hydrazone (CCCP), carbonyl cyanide m-trifluoromethoxyphenylhydrazone (FCCP), or dinitrophenol (DNP). Stepwise titration of uncouplers stimulates respiration up to or above the level of O\textsubscript{2} consumption rates in the OXPHOS-state; respiration is inhibited, however, above optimum uncoupler concentrations (Mitchell 2011). Data obtained with a single dose of uncoupler must be evaluated with caution, particularly when a fixed uncoupler concentration is used in studies exploring a treatment or disease that may alter the mitochondrial content or mitochondrial sensitivity to inhibition by uncouplers. The effect on ET-capacity of the reversed function of F-ATPase (\( J_{\text{p}} \); Figure 5C) can be evaluated in the presence and absence of extramitochondrial ATP.

2.5.4. ROX state and Rox: Besides the three fundamental coupling states of mitochondrial preparations, the state of residual O\textsubscript{2} consumption, ROX, is relevant to assess respiratory function (Figure 1). ROX is not a coupling state. The rate of residual oxygen consumption, Rox, is defined as O\textsubscript{2} consumption due to oxidative reactions measured after inhibition of ET—with retorone, malonic acid and antimycin A. Cyanide and azide inhibit not only CIV but catalase and several peroxidases involved in Rox. High concentrations of antimycin A, but not rotenone or cyanide, inhibit peroxisomal acyl-CoA oxidase and D-amino acid oxidase (Vamecq et al. 1987). Rox represents a baseline that is used to correct respiration measured in defined coupling states. Rox-corrected \( L, P \) and \( E \) not only lower the values of total fluxes, but also change the flux control ratios \( L/P \) and \( L/E \). Rox is not necessarily equivalent to non-
mitochondrial reduction of O₂, considering O₂-consuming reactions in mitochondria that are not related
to ET—such as O₂ consumption in reactions catalyzed by monoamine oxidases (type A and B),
monoxygenases (cytochrome P450 monoxygenases), dioxygenase (sulfur dioxygenase and
trimethyllysine dioxygenase), and several hydroxylases. Even isolated mitochondrial fractions,
especially those obtained from liver, may be contaminated by peroxisomes. This fact makes the exact
determination of mitochondrial O₂ consumption and mitochondria-associated generation of reactive
oxygen species complicated (Schönfeld et al. 2009; Speijer 2016; Figure 2). The dependence of ROX-
linked O₂ consumption needs to be studied in detail together with non-ET enzyme activities, availability
of specific substrates, O₂ concentration, and electron leakage leading to the formation of reactive oxygen
species.

2.5.5. Quantitative relations: E may exceed or be equal to P. E > P is observed in many types
of mitochondria, varying between species, tissues and cell types (Gnaiger 2009). E-P is the excess ET-
capacity pushing the phosphorylation-flux (Figure 2C) to the limit of its capacity of utilizing the
protonmotive force. In addition, the magnitude of E-P depends on the tightness of respiratory coupling
or degree of uncoupling, 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-pathway, under conditions when E remains constant but P declines relative to controls
(Figure 4). Substrate cocktails supporting simultaneous convergent electron transfer to the Q-junction
for reconstitution of TCA cycle function establish pathway control states with high ET-capacity, and
consequently increase the sensitivity of the E-P assay.

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 insufficient uncoupler concentrations; (3) using
high uncoupler concentrations which inhibit ET (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 ET-capacity is overestimated if non-saturating [ADP] or [Pi] are
used. See State 3 in the next section.

The net OXPHOS-capacity is calculated by subtracting L from P (Figure 4). The net P»/O₂ equals
P»/(P-L), wherein 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—by titration of an ET inhibitor (Divakaruni and Brand 2011).
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 O₂ flux measured in the OXPHOS and LEAK states. P-L is the upper limit
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 (Figure 4).

The rates of LEAK respiration and OXPHOS capacity depend on (1) the tightness of coupling
under the influence of the respiratory uncoupling mechanisms (Figure 3), and (2) the coupling
stoichiometry, which varies as a function of the substrate type undergoing oxidation in ET-pathways
with either two or three coupling sites (Figure 2B). When cocktails with NADH-linked substrates and
succinate are used, the relative contribution of ET-pathways with three or two coupling sites cannot be
controlled experimentally, is difficult to determine, and may shift in transitions between LEAK-,
OXPHOS- and ET-states (Gnaiger 2014). Under these experimental conditions, we cannot separate the
tightness of coupling versus coupling stoichiometry as the mechanisms of respiratory control in the shift
of L/P ratios. The tightness of coupling and fully coupled O₂ flux, P-L (Table 2), therefore, are obtained
from measurements of coupling control of LEAK respiration, OXPHOS- and ET-capacities in well
defined pathway states, using either pyruvate and malate as substrates or the classical succinate and
rotenone substrate-inhibitor combination (Figure 2B).

2.5.6. The steady-state: Mitochondria represent a thermodynamically open system in non-
equilibrium states of biochemical energy transformation. State variables (protonmotive force; redox
states) and metabolic rates (fluxes) are measured in defined mitochondrial respiratory states. Steady-
states can be obtained only in open systems, in which changes by internal transformations, e.g., O₂
consumption, are instantaneously compensated for by external fluxes, e.g., O₂ supply, preventing a
change of O₂ concentration in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored
in closed systems satisfy the criteria of pseudo-steady states for limited periods of time, when changes
in the system (concentrations of O₂, fuel substrates, ADP, Pᵢ, H⁺) do not exert significant effects on
metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require respiratory media with sufficient buffering capacity and substrates maintained at kinetically-saturating concentrations, and thus depend on the kinetics of the processes under investigation.

2.6. 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. States and rates are not specifically distinguished in this nomenclature.

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

<table>
<thead>
<tr>
<th>State</th>
<th>[O₂] ADP level</th>
<th>Substrate level</th>
<th>Respiration rate</th>
<th>Rate-limiting substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;0 low</td>
<td>low</td>
<td>slow</td>
<td>ADP</td>
</tr>
<tr>
<td>2</td>
<td>&gt;0 high</td>
<td>~0</td>
<td>slow</td>
<td>substrate</td>
</tr>
<tr>
<td>3</td>
<td>&gt;0 high</td>
<td>high</td>
<td>fast</td>
<td>respiratory chain</td>
</tr>
<tr>
<td>4</td>
<td>&gt;0 low</td>
<td>high</td>
<td>slow</td>
<td>ADP</td>
</tr>
<tr>
<td>5</td>
<td>0 high</td>
<td>high</td>
<td>0</td>
<td>oxygen</td>
</tr>
</tbody>
</table>

2.6.1. State 1 is obtained after addition of isolated mitochondria to air-saturated isosmotic/isotonic respiration medium containing P₄, but no fuel substrates and no adenylates, i.e., AMP, ADP, ATP.

2.6.2. 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 exhausted endogenous fuel substrate availability (Table 3). If addition of specific inhibitors of respiratory complexes—such as rotenone—does not cause a further decline of O₂ flux, State 2 is equivalent to the ROX state (See below.). If inhibition is observed, undefined endogenous fuel substrates are a confounding factor of pathway control, contributing to the effect of subsequently externally added substrates and inhibitors. In contrast to the original protocol, an alternative sequence of titration steps is frequently applied, in which the alternative ‘State 2’ has an entirely different meaning, when this second state is induced by addition of fuel substrate without ADP or ATP (LEAK-state; in contrast to State 2 defined in Table 1 as a ROX state). Some researchers have called this condition as “pseudostate 4” because it has no significant concentrations of adenine nucleotides and hence it is not a near-physiological condition, although it should be used for calculating the net OXPHOS-capacity, P-L.

2.6.3. 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 chamber. Repeated ADP titration re-establishes State 3 at ‘high ADP’. Starting at O₂ concentrations near air-saturation (193 or 238 µM O₂ at 37 °C or 25 °C and sea level at 1 atm or 101.32 kPa, and an oxygen solubility of respiration medium at 0.92 times that of pure water; Forstner and Gnaiger 1983), the total ADP concentration added must be low enough (typically 100 to 300 µM) to allow phosphorylation to ATP at a coupled O₂ flux that does not lead to O₂ depletion during the transition to State 4. In contrast, kinetically-saturating ADP concentrations usually are 10-fold higher than 'high ADP', e.g., 2.5 mM in isolated mitochondria. The abbreviation State 3u is occasionally 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 ET-capacity (noncoupled).
2.6.4. State 4 is a LEAK-state that is obtained only if the mitochondrial preparation is intact and well-coupled. Depletion of ADP by phosphorylation to ATP causes a decline of O₂ flux in the transition from State 3 to State 4. Under the conditions of State 4, a maximum protonmotive force and high ATP/ADP ratio are maintained. The gradual decline of \( \Delta p/o_2 \), towards diminishing [ADP] at State 4 must be taken into account for calculation of P\(s\)/O₂ ratios (Gnaiger 2001). State 4 respiration, \( L_T \) (Table 1), reflects intrinsic proton leak and ATP hydrolysis activity. O₂ flux in State 4 is an overestimation of LEAK-respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP, \( J_{0p} \), which stimulates respiration coupled to phosphorylation, \( J_P > 0 \). Some degree of mechanical disruption and loss of mitochondrial integrity allows the exposed mitochondrial F-ATPases to hydrolyze the ATP synthesized by the fraction of coupled mitochondria. This can be tested by inhibition of the phosphorylation-pathway using oligomycin, ensuring that \( J_{0p} = 0 \) (State 4o). On the other hand, the State 4 respiration reached after exhaustion of added ADP is a more physiological condition (i.e., presence of ATP, ADP and even AMP). Sequential ADP titrations re-establish State 3, followed by State 3 to State 4 transitions while sufficient O₂ is available. Anoxia may be reached, however, before exhaustion of ADP (State 5).

2.6.5. State 5 is the state after exhaustion of O₂ in a closed respirometric chamber. Diffusion of O₂ from the surroundings into the aqueous solution may be a confounding factor preventing complete anoxia (Gnaiger 2001). Chance and Williams (1955) provide an alternative definition of State 5, which gives it the different meaning of ROX versus anoxia: ‘State 5 may be obtained by antimycin A treatment or by anaerobiosis’.

In Table 3, only States 3 and 4 are coupling control states, with the restriction that rates in State 3 may be limited kinetically by non-saturating ADP concentrations.

2.7. 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 O₂, e.g., starvation and hypoxia; (4) the protonmotive force, redox states, flux–force relationships, coupling and efficiency; (5) Ca²⁺ and other ions including H⁺; (6) inhibitors, e.g., nitric oxide or intermediary metabolites such as oxaloacetate; (7) signalling pathways and regulatory proteins, e.g., insulin resistance, transcription factor hypoxia inducible 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). Mitochondria are targeted directly by hormones, e.g., progesterone and glucocorticoids, which affect 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 individuals; age; biological sex, and hormone concentrations; life style including exercise and nutrition; and environmental issues including thermal, atmospheric, toxic 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.

Lack of control by a metabolic pathway, e.g., phosphorylation-pathway, means that there will be no response to a variable activating it, e.g., [ADP]. The reverse, however, is not true as the absence of a response to [ADP] does not exclude the phosphorylation-pathway from having some degree of control. The degree of control of a component of the OXPHOS-pathway on an output variable—such as O₂ flux, will in general be different from the degree of control on other outputs—such as phosphorylation-flux or proton leak flux. Therefore, it is necessary to be specific as to which input and output are under consideration (Fell 1997).
Respiratory control refers to the ability of mitochondria to adjust O₂ flux in response to external control signals by engaging various mechanisms of control and regulation. Respiratory control is monitored in a mitochondrial preparation under conditions defined as respiratory states, preferentially under near-physiological conditions of temperature, pH and medium ionic composition, to generate data of higher biological relevance. When phosphorylation of ADP to ATP is stimulated or depressed, an increase or decrease is observed in electron transfer measured as O₂ flux in respiratory coupling states of intact mitochondria (‘controlled states’ in the classical terminology of bioenergetics). Alternatively, coupling of electron transfer with phosphorylation is diminished by uncouplers. The corresponding coupling control state is characterized by a high respiratory rate without control by Pₚ (noncoupled or ‘uncontrolled state’).

3. What is a rate?

The term rate is not adequately defined to be useful for reporting data. Normalization of ‘rates’ leads to a diversity of formats. Application of common and defined units is required for direct transfer of reported results into a database. The second [s] is the SI unit for the base quantity time. It is also the standard time-unit used in solution chemical kinetics. The inconsistency of the meanings of rate becomes apparent when considering Galileo Galilei’s famous principle, that ‘bodies of different weight all fall at the same rate (have a constant acceleration)’ (Coopersmith 2010). A rate may be an extensive quantity, which is a flow, \( J \), when expressed per object (per number of cells or organisms) or per chamber (per system). ‘System’ is defined as the open or closed chamber of the measuring device. A rate is a flux, \( J \), when expressed as a size-specific quantity (Figure 6A; Box 2).

- **Extensive quantities**: An extensive quantity increases proportionally with system size. For example, mass and volume are extensive quantities. Flow is an extensive quantity. The magnitude of an extensive quantity is completely additive for non-interacting subsystems. The magnitude of these quantities depends on the extent or size of the system (Cohen et al. 2008).

- **Size-specific quantities**: ‘The adjective specific before the name of an extensive quantity is often used to mean divided by mass’ (Cohen et al. 2008). In this system-paradigm, mass-specific flux is flow divided by mass of the system (the total mass of everything within the measuring chamber or reactor). Rates are frequently expressed as volume-specific flux. A mass-specific or volume-specific quantity is independent of the extent of non-interacting homogenous subsystems. Tissue-specific quantities (related to the sample in contrast to the system) are of fundamental interest in the field of comparative mitochondrial physiology, where specific refers to the type of the sample rather than mass of the system. The term specific, therefore, must be clarified; sample-specific, e.g., muscle mass-specific normalization, is distinguished from system-specific quantities (mass or volume; Figure 6).

- **Intensive quantities**: In contrast to size-specific properties, forces are intensive quantities defined as the change of an extensive quantity per advancement of an energy transformation (Gnaiger 1993b).

\( N_X \) and \( m_X \) indicate the number format and mass format, respectively, for expressing the quantity of a sample \( X \). When different formats are indicated in symbols of derived quantities, the format \( (N, m) \) is shown as a subscript (underlined italic), as in \( I_{O_{2\text{in}}X} \) and \( J_{O_{2\text{in}}X} \). Oxygen flow and flux are expressed in the molar format, \( n_{O_2} \) [mol], but in the volume format, \( V_{O_2} \) [m³] in ergometry. For mass-specific flux these formats can be distinguished as \( J_{O_{2\text{in}}X} \) and \( J_{O_{2\text{in}}X} \), respectively. Further examples are given in Figure 6 and Table 4.
Figure 6. Flow and flux, and normalization in structure-function analysis

(A) When expressing metabolic ‘rate’ measured in a chamber, a fundamental distinction is made between relating the rate to the experimental sample (left) or chamber (right). The different meanings of rate need to be specified by the chosen normalization. Left: Results are expressed as mass-specific flux, \( J_{mx} \), per mg protein, dry or wet mass. Cell volume, \( V_{ce} \), may be used for normalization (volume-specific flux, \( J_{V,ce} \)). Right: Flow per chamber, \( I \), or flux per chamber volume, \( J_{V} \), are merely reported for methodological reasons.

(B) \( O_2 \) flow per cell, \( I_{O_2}/N_{ce} \), is the product of mitochondria-specific flux, \( mt \)-density and mass per cell. Unstructured analysis: performance is the product of mass-specific flux, \( J_{O_2}/M_{X} \) [mol∙s\(^{-1}\)∙kg\(^{-1}\)], and size (mass per cell). Structured analysis: performance is the product of mitochondrial function (mt-specific flux) and structure (mt-content).

Modified from Gnaiger (2014). For further details see Table 4.

Box 2: Metabolic flows and fluxes: vectoral, vectorial, and scalar

In a generalization of electrical terms, flow as an extensive quantity (\( I \); per system) is distinguished from flux as a size-specific quantity (\( J \); per system size). Flows, \( I_{a} \), are defined for all transformations as extensive quantities. Electric charge per unit time is electric flow or current, \( I_{a} = dQ_{a}/dt \) [A≡ C∙s\(^{-1}\)]. When dividing \( I_{a} \) by size of the system (cross-sectional area of a ‘wire’), we obtain flux as a size-specific quantity, which is the current density (surface-density of flow) perpendicular to the direction of flux, \( J_{a} = I_{a}/A \) [A∙m\(^{-2}\)] (Cohen et al. 2008). Fluxes with spatial geometric direction and magnitude are vectors. Vector and scalar fluxes are related to flows as \( J_{a} = I_{a}/A \) [mol∙s\(^{-1}\)∙m\(^{-2}\)] and \( J_{a} = I_{a}/V \) [mol∙s\(^{-1}\)∙m\(^{-3}\)], expressing flux as an area-specific vector or volume-specific vectorial or scalar quantity, respectively (Gnaiger 1993b). We use the metre–kilogram–second–ampere (MKSA) international system of units (SI) for general cases ([m], [kg], [s] and [A]), with decimal SI prefixes for specific applications (Table 4).

We suggest to define: (1) vectoral fluxes, which are translocations as functions of gradients with direction in geometric space in continuous systems; (2) vectorial fluxes, which describe translocations in discontinuous systems and are restricted to information on compartmental differences (transmembrane proton flux); and (3) scalar fluxes, which are transformations in a homogenous system (catabolic O\(_2\) flux, \( J_{kO_2} \)).
4. Normalization of rate per sample

The challenges of measuring mitochondrial respiratory flux are matched by those of normalization. Normalization (Table 4) is guided by physicochemical principles, methodological considerations, and conceptual strategies (Figure 6).

Table 4. Sample concentrations and normalization of flux.

<table>
<thead>
<tr>
<th>Expression</th>
<th>Symbol</th>
<th>Definition</th>
<th>Unit</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>identity of sample</td>
<td>$X$</td>
<td>object: cell, tissue, animal, patient</td>
<td></td>
</tr>
<tr>
<td>number of sample entities $X$</td>
<td>$N_X$</td>
<td>number of objects</td>
<td>$x$</td>
<td>1</td>
</tr>
<tr>
<td>mass of sample $X$</td>
<td>$m_X$</td>
<td>kg</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>mass of object $X$</td>
<td>$M_X = m_X N_X^{-1}$</td>
<td>kg·x$^{-1}$</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>amount of mt-elementary components</td>
<td>$mtE$</td>
<td>quantity of mt-marker</td>
<td>mtEU</td>
</tr>
<tr>
<td>Concentrations</td>
<td>object number concentration</td>
<td>$C_{gX} = N_X V^{-1}$</td>
<td>x·m$^{-3}$</td>
<td>3</td>
</tr>
<tr>
<td>sample mass concentration</td>
<td>$C_{gX} = m_X V^{-1}$</td>
<td>kg·m$^{-3}$</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>mitochondrial concentration</td>
<td>$C_{mtE} = mTE V^{-1}$</td>
<td>mtEU·m$^{-3}$</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>specific mitochondrial density</td>
<td>$D_{mtE} = mTE m_X^{-1}$</td>
<td>mtEU·kg$^{-1}$</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>mitochondrial content, $mtE$ per object $X$</td>
<td>$mtE_{gX} = mTE N_X^{-1}$</td>
<td>mtEU·x$^{-1}$</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>O$_2$ flow and flux</td>
<td>flow, system</td>
<td>$I_{O_2}$</td>
<td>internal flow</td>
<td>mol·s$^{-1}$</td>
</tr>
<tr>
<td>volume-specific flux</td>
<td>$J_{VO_2} = I_{O_2} V^{-1}$</td>
<td>mol·s$^{-1}$·m$^{-3}$</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>flow per object $X$</td>
<td>$I_{O_2 X} = J_{VO_2} C_{gX}^{-1}$</td>
<td>mol·s$^{-1}$·x$^{-1}$</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>mass-specific flux</td>
<td>$J_{grinE} = I_{O_2 X} C_{gE}^{-1}$</td>
<td>mol·s$^{-1}$·kg$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mt-marker-specific flux</td>
<td>$J_{grinmtE} = J_{VO_2} C_{mtE}$</td>
<td>mol·s$^{-1}$·mtEU$^{-1}$</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

1 The unit x for a number is not used by IUPAC. To avoid confusion, the units [kg·x$^{-1}$] and [kg]
distinguish the mass per object from the mass of a sample that may contain any number of objects.

2 Units are given in the MKSA system (Box 2). 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.

3 In case of cells (sample $X =$ cells), the object number concentration is $C_{N_e} = N_e V^{-1}$, and volume may be expressed in [dm$^3$ = L] or [cm$^3$ = mL]. See Table 5 for different object types.

4 mt-concentration is an experimental variable, dependent on sample concentration: (1) $C_{mtE} = mTE V^{-1}$; (2) $C_{mtE} = mTE C_{NX}$; (3) $C_{mtE} = mTE D_{mtE}$.

5 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.

6 $mtE_{NX} = mTE N_X^{-1} = C_{mtE} C_{NX}^{-1}$.

7 O$_2$ can be replaced by other chemicals to study different reactions, e.g., ATP, H$_2$O$_2$, or vesicular compartmental translocations, e.g., Ca$^{2+}$. 

8 $I_{02}$ and $V$ are defined per instrument chamber as a system of constant volume (and constant temperature), which may be closed or open. $I_{02}$ is abbreviated for $I_{02}$, 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, $\nu_{02} = -1$. $I_{02} = \frac{dN_{O2}}{dt}V_{Y0}^{-1}$. If $r$ includes all chemical reactions in which O$_2$ participates, then $dN_{O2} = dN_{O2} - dN_{O2}$, where $dN_{O2}$ is the change in the amount of O$_2$ in the instrument chamber and $dN_{O2}$ is the amount of O$_2$ added externally to the system. At steady state, by definition $dN_{O2} = 0$, hence $dN_{O2} = -dN_{O2}$. Note that in this context ‘external’, e, refers to the system, whereas in Figure 1 ‘external’, ext, refers to the organism.

9 $I_{02}/O_2$ is an experimental variable, expressed per volume of the instrument chamber.

10 $I_{02}/O_2$ is a physiological variable, depending on the size of entity $X$.

11 There are many ways to normalize for a mitochondrial marker, that are used in different experimental approaches: (1) $J_{O2\text{int}} = I_{02}/C_{\text{mit}}$; (2) $J_{O2\text{ext}} = I_{02}/C_{\text{m}}^{1-1}D_{\text{mit}}^{-1} = J_{O2\text{int}}D_{\text{mit}}^{-1}$; (3) $J_{O2\text{int}} = I_{02}/C_{\text{m}}^{1-1}C_{\text{mit}}^{-1}mtE_{\text{mit}}^{-1}$; (4) $J_{O2\text{max}} = I_{02}/mtE^{-1}$. The mt-elementary unit [mtEU] varies depending on the mt-marker.

Table 5. Sample types, $X$, abbreviations, and quantification.

<table>
<thead>
<tr>
<th>Identity of sample</th>
<th>$X$</th>
<th>$N_X$</th>
<th>Mass$^a$</th>
<th>Volume</th>
<th>mt-Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>isolated mitochondria</td>
<td>int</td>
<td>$m_{mit}$</td>
<td>$V_{mit}$</td>
<td>$mtE$</td>
<td></td>
</tr>
<tr>
<td>tissue homogenate</td>
<td>thom</td>
<td>$m_{thom}$</td>
<td>$mtE_{thom}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>permeabilized tissue</td>
<td>pti</td>
<td>$m_{pti}$</td>
<td>$mtE_{pti}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>permeabilized fibre</td>
<td>pfi</td>
<td>$m_{pti}$</td>
<td>$mtE_{pti}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>permeabilized cell</td>
<td>pce</td>
<td>$N_{pce}$</td>
<td>$M_{pce}$</td>
<td>$V_{pce}$</td>
<td>$mtE_{pce}$</td>
</tr>
<tr>
<td>ce</td>
<td>$N_{ce}$</td>
<td>$M_{ce}$</td>
<td>$V_{ce}$</td>
<td>$mtE_{ce}$</td>
<td></td>
</tr>
<tr>
<td>dead cell</td>
<td>dce</td>
<td>$N_{dce}$</td>
<td>$M_{dce}$</td>
<td>$V_{dce}$</td>
<td></td>
</tr>
<tr>
<td>organism</td>
<td>org</td>
<td>$N_{org}$</td>
<td>$M_{org}$</td>
<td>$V_{org}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Instead of mass, the wet weight or dry weight is frequently stated, $W_w$ or $W_d$. $m_X$ is mass of the sample [kg], $M_X$ is mass of the object [kg x$^{-1}$] (Table 4).

$^b$ Total cell count, $N_{ce} = N_{vce} + N_{dce}$.

4.1. Flow: per object

4.1.1. Number concentration, $C_{X\text{X}}$: Normalization per sample concentration is routinely required to report respiratory data. $C_{X\text{X}}$ is the experimental number concentration of sample $X$. In the case of animals, e.g., nematodes, $C_{X\text{X}} = N_X/V$ [x L$^{-1}$], where $N_X$ is the number of organisms in the chamber. Similarly, the number of cells per chamber volume is the number concentration of permeabilized or intact cells $C_{vce} = N/v/V$ [x L$^{-1}$], where $N_{vce}$ is the number of cells in the chamber (Table 4).

4.1.2. Flow per object, $I_{O2\text{X}}$: O$_2$ flow per cell is calculated from volume-specific O$_2$ flux, $J_{O2\text{X}}$ [nmol s$^{-1}$ L$^{-1}$] (per V of the measurement chamber [L]), divided by the number concentration of cells.

The total cell count is the sum of viable and dead cells, $N_{ce} = N_{vce} + N_{dce}$ (Table 5). The cell viability index, $VI = N_{vce}/N_{ce}$, is the ratio of viable cells ($N_{vce}$ before experimental permeabilization) per total cell count. After experimental permeabilization, all cells are permeabilized, $N_{pti} = N_{ce}$. The cell viability index can be used to normalize respiration for the number of cells that have been viable before experimental permeabilization, $I_{O2\text{max}} / N_{vce} = I_{O2\text{max}} / VI$, considering that mitochondrial respiratory dysfunction in dead cells should be eliminated as a confounding factor.

The complexity changes when the object is a whole organism studied as an experimental model.

The scaling law in respiratory physiology reveals a strong interaction between O$_2$ flow and individual body mass: basal metabolic rate (flow) does not increase linearly with body mass, whereas maximum mass-specific O$_2$ flux, $V_{O2\text{max}}$ or $V_{O2\text{peak}}$, is approximately constant across a large range of individual body mass (Weibel and Hoppeler 2005). Individuals, breeds and species, however, deviate substantially from this relationship. $V_{O2\text{peak}}$ of human endurance athletes is 60 to 80 mL O$_2$ min$^{-1}$ kg$^{-1}$ body mass, converted to $J_{O2\text{peak}}/\text{kg body mass}$ of 45 to 60 nmol s$^{-1}$ g$^{-1}$ (Gnaiger 2014; Table 6).
4.2. Size-specific flux: per sample size

4.2.1. Sample concentration, $C_{mX}$: Considering permeabilized tissue, homogenate or cells as the sample, $X$, the sample mass is $m_X$ [mg], which is frequently measured as wet or dry weight, $W_w$ or $W_d$ [mg], respectively, or as amount of protein, $m_{Protein}$. The sample concentration is the mass of the subsample per volume of the measurement chamber, $C_{mX} = m_X / V \text{ [g}$·$\text{L}^{-1} = \text{mg}$·$\text{mL}^{-1}]$. $X$ is the type of sample—isolated mitochondria, tissue homogenate, permeabilized fibres or cells (Table 5).

4.2.2. Size-specific flux: Cellular $O_2$ flow can be compared between cells of identical size. To take into account changes and differences in cell size, normalization is required to obtain cell size-specific or mitochondrial marker-specific $O_2$ flux (Renner et al. 2003).

- **Mass-specific flux, $J_{O2,mX}$** [mol·s$^{-1}$·kg$^{-1}$]: Mass-specific flux is obtained by expressing respiration per mass of sample, $m_X$ [mg]. Flow per cell is divided by mass per cell, $J_{O2,cell} = I_{O2,murea} / M_{Nce}$. Or chamber volume-specific flux, $J_{V,O2}$, is divided by mass concentration of $X$ in the chamber, $J_{V,O2,X} = J_{V,O2} / C_{mX}$.

- **Cell volume-specific flux, $J_{V,OX}$** [mol·s$^{-1}$·m$^{-3}$]: Sample volume-specific flux is obtained by expressing respiration per volume of sample. For example, in the case of using cells as sample will be the volume of cells added to the chamber (Figure 6).

If size-specific $O_2$ flux is constant and independent of sample size, then there is no interaction between the subsystems. For example, a 1.5 mg and a 3.0 mg muscle sample respire at identical mass-specific flux. Mass-specific $O_2$ flux, however, may change with the mass of a tissue sample, cells or isolated mitochondria in the measuring chamber, in which the nature of the interaction becomes an issue. Therefore, cell density must be optimized, particularly in experiments carried out in wells, considering the confluency of the cell monolayer or clumps of cells (Salabei et al. 2014).

4.3. Marker-specific flux: per mitochondrial content

Tissues can contain multiple cell populations that may have distinct mitochondrial subtypes. Mitochondria undergo dynamic fission and fusion cycles, and can exist in multiple stages and sizes that 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, depending on the isolation protocols utilized (e.g., centrifugation speed). This possible bias should be taken into account when planning experiments using isolated mitochondria. Different sizes of mitochondria are enriched at specific centrifugation speeds, which can be used strategically for isolation of mitochondrial subpopulations.

Part of the mitochondrial content of a tissue is lost during preparation of isolated mitochondria. The fraction of isolated mitochondria obtained from a tissue sample is expressed as mitochondrial recovery. At a high mitochondrial recovery the fraction of isolated mitochondria is more representative of the total mitochondrial population than in preparations characterized by low recovery. Determination of the mitochondrial recovery and yield is based on measurement of the concentration of a mitochondrial marker in the stock of isolated mitochondria, $C_{mtE,stock}$, and crude tissue homogenate, $C_{mtE,thom}$, which simultaneously provides information on the specific mitochondrial density in the sample, $D_{mtE}$ (Table 4).

When discussing concepts on normalization, it is essential to consider the question of the study. If the study aims at comparing tissue performance—such as the effects of a treatment on a specific tissue, then normalization for tissue mass or protein content is appropriate. However, if the aim is to find differences on mitochondrial function independent of mitochondrial density (Table 4), then normalization to a mitochondrial marker is imperative (Figure 6). One cannot assume that quantitative changes in various markers—such as mitochondrial proteins—necessarily occur in parallel with one another. It should be established 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 standardization on normalization for entry into a databank.

4.3.1. Mitochondrial concentration, $C_{mt}$, and mitochondrial markers: Mitochondrial organelles comprise a dynamic cellular reticulum in various states of fusion and fission. Hence, the definition of an “amount” of mitochondria is often misconceived: mitochondria cannot be counted reliably as a number of occurring elementary components. Therefore, quantification of the “amount” of
mitochondria depends on the measurement of chosen mitochondrial markers. ‘Mitochondria are the 1154 structural and functional elementary units of cell respiration’ (Gnaiger 2014). The quantity of a 1155 mitochondrial marker can reflect the amount of mitochondrial elementary components, \( mtE \), expressed 1156 in various mitochondrial elementary units [\( mtEU \)] specific for each measured \( mt \)-marker (Table 4). 1157 However, since mitochondrial quality may change in response to stimuli — particularly in mitochondrial 1158 dysfunction (Campos et al. 2017) and after exercise training (Pesta et al. 2011) and during aging (Daum 1159 et al. 2013) — some markers can vary while others are unchanged: (1) Mitochondrial volume and 1160 membrane area are structural markers, whereas mitochondrial protein mass is commonly used as a 1161 marker for isolated mitochondria. (2) Molecular and enzymatic mitochondrial markers (amounts or 1162 activities) can be selected as matrix markers, e.g., citrate synthase activity, mtDNA; mtMMarkers, e.g., 1163 cytochrome \( c \) oxidase activity, \( aa_3 \) content, cardiolipin, or mtOM-markers, e.g., the voltage-dependent 1164 anion channel (VDAC), TOM20. (3) Extending the measurement of mitochondrial marker enzyme 1165 activity to mitochondrial pathway capacity, ET- or OXPHOS-capacity can be considered as an 1166 integrative functional mitochondrial marker.

Depending on the type of mitochondrial marker, the mitochondrial elementary component, \( mtE \), 1167 is expressed in marker-specific units. Mitochondrial concentration in the measurement chamber and the 1168 tissue of origin are quantified as (1) a quantity for normalization in functional analyses, \( C_{mtE} \), and (2) a 1169 physiological output that is the result of mitochondrial biogenesis and degradation, \( D_{mtE} \), respectively 1170 (Table 4). It is recommended, therefore, to distinguish experimental mitochondrial concentration, \( C_{mtE} \) 1171 = \( mtE/V \) and physiological mitochondrial density, \( D_{mtE} = mtE/mX \). Then mitochondrial density is the 1172 amount of mitochondrial elementary components per mass of tissue, which is a biological variable 1173 (Figure 6). The experimental variable is mitochondrial density multiplied by sample mass concentration 1174 in the measuring chamber, \( C_{mtE} = D_{mtE}C_{mX} \), or mitochondrial content multiplied by sample number 1175 concentration, \( C_{mtE} = mtEXC_{mX} \) (Table 4).

### 4.3.2. \( mt \)-Marker-specific flux, \( J_{O_{2}mtE} \)

Volume-specific metabolite \( O_2 \) flux depends on: (1) the sample concentration in the volume of the instrument chamber, \( C_{mX} \) or \( C_{mX} \); (2) the mitochondrial density in the sample, \( D_{mtE} = mtE/mX \) or \( mtE = mtE/mX \) and (3) the specific mitochondrial activity or 1176 performance per elementary mitochondrial unit, \( J_{O_{2}mtE} = J_{\Delta \psi}/C_{mtE} \) [\( \text{mol} \text{s}^{-1} \text{mtEU}^{-1} \)] (Table 4). 1177 Obviously, the numerical results for \( J_{O_{2}mtE} \) vary with the type of mitochondrial marker chosen for 1178 measurement of \( mtE \) and \( C_{mtE} = mtE/V \) [\( \text{mtEU} \text{m}^{-3} \)].

Different methods are implicated in the quantification of mitochondrial markers and have 1179 different strengths. Some problems are common for all mitochondrial markers, \( mtE \): (I) Accuracy of 1180 measurement is crucial, since even a highly accurate and reproducible measurement of \( O_2 \) flux results 1181 in an inaccurate and noisy expression if normalized by a biased and noisy measurement of a 1182 mitochondrial marker. This problem is acute in mitochondrial respiration because the denominators used 1183 (the mitochondrial markers) are often small moieties of which accurate and precise determination is 1184 difficult. This problem can be avoided when \( O_2 \) fluxes measured in substrate-uncoupler-inhibitor 1185 titration protocols are normalized for flux in a defined respiratory reference state, which is used as an 1186 internal marker and yields flux control ratios, \( FCRs \). \( FCRs \) are independent of externally measured 1187 markers and, therefore, are statistically robust, considering the limitations of ratios in general (Jasienski 1188 and Bazzaz 1999). \( FCRs \) indicate qualitative changes of mitochondrial respiratory control, with highest 1189 quantitative resolution, separating the effect of mitochondrial density or concentration on \( J_{O_{2}mtE} \) and 1190 \( I_{O_{2}mX} \) from that of function per elementary mitochondrial marker, \( J_{O_{2}mtE} \) (Pesta et al. 2011; Gnaiger 1191 2014). (2) If mitochondrial quality does not change and only the amount of mitochondria varies as a 1192 determinant of mass-specific flux, any marker is equally qualified in principle; then in practice selection 1193 of the optimum marker depends only on the accuracy and precision of measurement of the mitochondrial 1194 marker. (3) If mitochondrial flux control ratios change, then there may not be any best mitochondrial 1195 marker. In general, measurement of multiple mitochondrial markers enables a comparison and 1196 evaluation of normalization for a variety of mitochondrial markers. Particularly during postnatal 1197 development, the activity of marker enzymes — such as cytochrome \( c \) oxidase and citrate synthase — 1198 follows different time courses (Drahota et al. 2004). Evaluation of mitochondrial markers in healthy 1199 controls is insufficient for providing guidelines for application in the diagnosis of pathological states 1200 and specific treatments.

In line with the concept of the respiratory control ratio (Chance and Williams 1955a), the most 1201 readily used normalization is that of flux control ratios and flux control factors (Gnaiger 2014). Selection 1202 of the state of maximum flux in a protocol as the reference state has the advantages of: (1) internal
normalization; (2) statistically validated linearization of the response in the range of 0 to 1; and (3)
consideration of maximum flux for integrating a large number of elementary steps in the OXPHOS- or
ET-pathways. This reduces the risk of selecting a functional marker that is specifically altered by the
treatment or pathology, yet increases the chance that the highly integrative pathway is disproportionately
affected, e.g., the OXPHOS- rather than ET-pathway in case of an enzymatic defect in the
phosphorylation-pathway. In this case, additional information can be obtained by reporting flux control
ratios based on a reference state which indicates stable tissue-mass specific flux.

Stereological determination of mitochondrial content via two-dimensional transmission electron
microscopy can have limitations due to the dynamics of mitochondrial size (Meinild Lundby et al.
2017). Accurate determination of three-dimensional volume by two-dimensional microscopy can be
both time consuming and statistically challenging (Larsen et al. 2012).

The validity of using mitochondrial marker enzymes (citrate synthase activity, CI to CIV amount
or activity) for normalization of flux is limited in part by the same factors that apply to flux control
ratios. Strong correlations between various mitochondrial markers and citrate synthase activity
(Reichmann et al. 1985; Boushel et al. 2007; Mogensen et al. 2007) are expected in a specific tissue of
healthy persons and in disease states not specifically targeting citrate synthase. Citrate synthase activity
is acutely modifiable by exercise (Tonkonogi et al. 1997; Leek et al. 2001). Evaluation of mitochondrial
markers related to a selected age and sex cohort cannot be extrapolated to provide recommendations for
normalization in respirometric diagnosis of disease, in different states of development and ageing,
different cell types, tissues, and species. mtDNA normalized to nDNA via qPCR is correlated to
functional mitochondrial markers including OXPHOS- and ET-capacity in some cases (Puntschart et al.
1995; Wang et al. 1999; Menshikova et al. 2006; Boushel et al. 2007; Ehinger et al. 2015), but lack of
such correlations have been reported (Menshikova et al. 2005; Schultz and Wiesner 2000; Pesta et al.
2011). Several studies indicate a strong correlation between cardiolipin content and increase in
mitochondrial function with exercise (Menshikova et al. 2005; Menshikova et al. 2007; Larsen et al.
2012; Faber et al. 2014), but it has not been evaluated as a general mitochondrial biomarker in disease.

With no single best mitochondrial marker, a good strategy is to quantify several different biomarkers to
minimize the decorrelating effects caused by diseases, treatments, or other factors. Determination of
multiple markers, particularly a matrix marker and a marker from the mtIM, allows tracking changes in
mitochondrial quality defined by their ratio.

5. Normalization of rate per system

5.1. Flow: per chamber

The experimental system (experimental chamber) is part of the measurement instrument, separated from
the environment as an isolated, closed, open, isothermal or non-isothermal system (Table 4). Reporting O₂ flows per respiratory chamber, \( I_{O_2} \) [nmol·s⁻¹], restricts the analysis to intra-experimental comparison of relative differences.

5.2. Flux: per chamber volume

5.2.1. System-specific flux, \( J_{V_{O_2}} \): We distinguish between (1) the system with volume \( V \) and mass \( m \) defined by the system boundaries, and (2) the sample or objects with volume \( V_x \) and mass \( m_x \) that are enclosed in the experimental chamber (Figure 6). Metabolic O₂ flow per object, \( I_{O_2}^{[O_2]} \), is the total O₂ flow in the system divided by the number of objects, \( N_x \), in the system. \( I_{O_2}^{[O_2]_x} \) increases as the mass of the object is increased. Sample mass-specific O₂ flux, \( J_{O_2}^{[O_2]_x} \) should be independent of the mass of the sample studied in the instrument chamber, but system volume-specific O₂ flux, \( J_{V_{O_2}} \) (per volume of the instrument chamber), increases in proportion to the mass of the sample in the chamber. Whereas \( J_{V_{O_2}} \) depends on mass-concentration of the sample in the chamber, it should be independent of the chamber (system) volume at constant sample mass. There are practical limitations to increase the mass-concentration of the sample in the chamber, when one is concerned about crowding effects and instrumental time resolution.

5.2.2. Advancement per volume: When the reactor volume does not change during the reaction, which is typical for liquid phase reactions, the volume-specific flux of a chemical reaction \( r \) is the time
derivative of the advancement of the reaction per unit volume, \( J_{\text{Y:B}} = \frac{dc_{B}}{dr} V^{-1} \) [(mol·s\(^{-1}\)·L\(^{-1}\)]. The rate of concentration change is \( dc_{B}/dt \) [(mol·L\(^{-1}\)·s\(^{-1}\)]. There is a difference between \( J_{\text{Y:O}_2} \) [mol·s\(^{-1}\)·L\(^{-1}\)] and (2) rate of concentration change [mol·L\(^{-1}\)·s\(^{-1}\)]. These merge to a single expression only in closed systems. In open systems, internal transformations (catabolic flux, \( O_2 \) consumption) are distinguished from external flux (such as \( O_2 \) supply). External fluxes of all substances are zero in closed systems. In a closed chamber \( O_2 \) consumption (internal flux of catabolic reactions \( k \)), \( I_{\text{O}_2} \) [mol·s\(^{-1}\)], causes a decline of the amount of \( O_2 \) in the system, \( n_{\text{O}_2} \) [nmol]. Normalization of these quantities for the volume of the system, \( V \) [L ≡ dm\(^3\)], yields volume-specific \( O_2 \) flux, \( J_{\text{V:O}_2} = I_{\text{O}_2}/V \) [nmol·s\(^{-1}\)·L\(^{-1}\)], and \( O_2 \) concentration, \( [O_2] \) or \( c_{O_2} = n_{O_2}/V \) [µmol·L\(^{-1}\) = μM = mmol·mL\(^{-1}\)]. Instrumental background \( O_2 \) flux is due to external flux into a non-ideal closed respirometer; then total volume-specific flux has to be corrected for instrumental background \( O_2 \) flux—\( O_2 \) diffusion into or out of the instrumental chamber. \( J_{\text{V:O}_2} \) is relevant mainly for methodological reasons and should be compared with the accuracy of instrumental resolution of background-corrected flux, e.g., ±1 nmol·s\(^{-1}\) (Gnaiger 2001). ‘Metabolic’ or catabolic indicates \( O_2 \) flux, \( J_{\text{O}_2} \), corrected for: (1) instrumental background \( O_2 \) flux; (2) chemical background \( O_2 \) flux due to autoxidation of chemical components added to the incubation medium; and (3) \( R_{\text{ox}} \) for \( O_2 \)-consuming side reactions unrelated to the catabolic pathway \( k \).

6. Conversion of units

Many different units have been used to report the \( O_2 \) consumption rate, OCR (Table 6). SI base units provide the common reference to introduce the theoretical principles (Figure 6), 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 7). Reporting data in SI units—including the mole [mol], coulomb [C], joule [J], and second [s]—should be encouraged, particularly by journals which propose the use of SI units.

**Table 6. Conversion of various formats and units used in respirometry and ergometry.** \( e \) is the number of electrons or reducing equivalents. \( z_B \) is the charge number of entity B.

<table>
<thead>
<tr>
<th>Format</th>
<th>1 Unit</th>
<th>Multiplication factor</th>
<th>SI-unit</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>ng·atom O·s(^{-1})</td>
<td>(2 ( e^- ))</td>
<td>0.5</td>
<td>nmol O(_2)·s(^{-1})</td>
</tr>
<tr>
<td>( n )</td>
<td>ng·atom O·min(^{-1})</td>
<td>(2 ( e^- ))</td>
<td>8.33</td>
<td>pmol O(_2)·s(^{-1})</td>
</tr>
<tr>
<td>( n )</td>
<td>natom O·min(^{-1})</td>
<td>(2 ( e^- ))</td>
<td>8.33</td>
<td>pmol O(_2)·s(^{-1})</td>
</tr>
<tr>
<td>( n )</td>
<td>nmol O(_2)·min(^{-1})</td>
<td>(4 ( e^- ))</td>
<td>16.67</td>
<td>pmol O(_2)·s(^{-1})</td>
</tr>
<tr>
<td>( n )</td>
<td>nmol O(_2)·h(^{-1})</td>
<td>(4 ( e^- ))</td>
<td>0.2778</td>
<td>pmol O(_2)·s(^{-1})</td>
</tr>
<tr>
<td>( V ) to ( \mu_l )</td>
<td>mL O(_2)·min(^{-1}) at STPD(^d)</td>
<td>0.744</td>
<td>μmol O(_2)·s(^{-1})</td>
<td>3</td>
</tr>
<tr>
<td>( e ) to ( \mu_l )</td>
<td>W = J/s at ~470 kJ/mol O(_2)</td>
<td>-2.128</td>
<td>μmol O(_2)·s(^{-1})</td>
<td>3</td>
</tr>
<tr>
<td>( e ) to ( \mu_l )</td>
<td>mA = mC·s(^{-1}) ((2\text{H}+ = 1))</td>
<td>10.36</td>
<td>nmol H(^+)·s(^{-1})</td>
<td>3</td>
</tr>
<tr>
<td>( e ) to ( \mu_l )</td>
<td>mA = mC·s(^{-1}) ((2\text{O}_2 = 4))</td>
<td>2.59</td>
<td>nmol O(_2)·s(^{-1})</td>
<td>3</td>
</tr>
<tr>
<td>( \eta ) to ( e )</td>
<td>nmol H(^+)·s(^{-1}) ((2\text{H}+ = 1))</td>
<td>0.09649</td>
<td>mA</td>
<td>3</td>
</tr>
<tr>
<td>( \eta ) to ( e )</td>
<td>nmol O(_2)·s(^{-1}) ((2\text{O}_2 = 4))</td>
<td>0.38594</td>
<td>mA</td>
<td>3</td>
</tr>
</tbody>
</table>

1. At standard temperature and pressure dry (STPD: 0 °C = 273.15 K and 1 atm = 101.325 kPa = 760 mmHg), the molar volume of an ideal gas, \( V_m \), and \( V_m:O_2 \) is 22.414 and 22.392 L·mol\(^{-1}\), respectively. Rounded to three decimal places, both values yield the conversion factor of 0.744.

2. For comparison at normal temperature and pressure dry (NTPD: 20 °C), \( V_m:O_2 \) is 24.038 L·mol\(^{-1}\). Note that the SI standard pressure is 100 kPa.

3. The multiplication factor is \( \eta_B/F \).
Table 7. Conversion of units with preservation of numerical values.

<table>
<thead>
<tr>
<th>Name</th>
<th>Frequently used unit</th>
<th>Equivalent unit</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>volume-specific flux, $J_{V,O_2}$</td>
<td>pmol·s⁻¹·mL⁻¹</td>
<td>nmol·s⁻¹·L⁻¹</td>
<td>1</td>
</tr>
<tr>
<td>cell-specific flow, $I_{O_2/cell}$</td>
<td>mmol·s⁻¹·L⁻¹</td>
<td>mol·s⁻¹·m⁻³</td>
<td></td>
</tr>
<tr>
<td>cell number concentration, $C_{Nec}$</td>
<td>pmol·s⁻¹·10⁶ cells</td>
<td>amol·s⁻¹·cell⁻¹</td>
<td>2</td>
</tr>
<tr>
<td>mass-specific flux, $J_{O_2/m}$</td>
<td>pmol·s⁻¹·10⁹ cells</td>
<td>zmol·s⁻¹·cell⁻¹</td>
<td>3</td>
</tr>
<tr>
<td>mitochondrial protein concentration, $C_{mol}$</td>
<td>10⁶ cells·mL⁻¹</td>
<td>10⁹ cells·L⁻¹</td>
<td></td>
</tr>
<tr>
<td>volume</td>
<td>0.1 mg·mL⁻¹</td>
<td>0.1 g·L⁻¹</td>
<td></td>
</tr>
<tr>
<td>catabolic power, $P_c$</td>
<td>pmol·s⁻¹·mg⁻¹</td>
<td>nmol·s⁻¹·g⁻¹</td>
<td>4</td>
</tr>
<tr>
<td>volume</td>
<td>μW·10⁻⁶ cells</td>
<td>pW·cell⁻¹</td>
<td>1</td>
</tr>
<tr>
<td>amount of substance concentration</td>
<td>1,000 L</td>
<td>m³ (1,000 kg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>dm³ (kg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mL</td>
<td>cm³ (g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>μL</td>
<td>mm³ (mg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fL</td>
<td>μm³ (pg)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>M = mol·L⁻¹</td>
<td>mol·dm⁻³</td>
<td></td>
</tr>
</tbody>
</table>

Although volume is expressed as m³ using the SI base unit, the litre [dm³] is a conventional unit of volume for concentrations and is used for most solution chemical kinetics. If one multiplies $I_{O_2/cell}$ by $C_{Nec}$, then the result will not only be the amount of O₂ [mol] consumed per time [s⁻¹] in one litre [L⁻¹], but also the change in O₂ concentration per second (for any volume of an ideally closed system). This is ideal for kinetic modeling as it blends with chemical rate equations where concentrations are typically expressed in mol·L⁻¹ (Wagner et al. 2011). In studies of multinuclear cells—such as differentiated skeletal muscle cells—it is easy to determine the number of nuclei but not the total number of cells. A generalized concept, therefore, is obtained by substituting cells by nuclei as the sample entity. This does not hold, however, for enucleated platelets.

For studies of cells, we recommend that respiration be expressed, as far as possible, as: (1) O₂ flux normalized for a mitochondrial marker, for separation of the effects of mitochondrial quality and content on cell respiration (this includes FCRs as a normalization for a functional mitochondrial marker); (2) O₂ 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) O₂ flow in units of atomole (10⁻¹⁸ mol) of O₂ consumed in a second by each cell [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 O₂ flow must be considered. For example, to estimate the volume-specific O₂ 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 O₂ [mol] consumed per time [s⁻¹] per unit volume [L⁻¹]. At an O₂ flow of 100 amol·s⁻¹·cell⁻¹ and a cell density of 10⁹ cells·L⁻¹ (10⁶ cells·mL⁻¹), the volume-specific O₂ flux is 100 nmol·s⁻¹·L⁻¹ (100 pmol·s⁻¹·mL⁻¹).

ET-capacity in human cell types including HEK 293, primary HUVEC and fibroblasts ranges from 50 to 180 amol·s⁻¹·cell⁻¹, measured in intact cells in the noncoupled state (see Gnaiger 2014). At 100 amol·s⁻¹·cell⁻¹ corrected for Rox, the current across the mitochondrial membranes, $I_{int}$, approximates 193 pA·cell⁻¹ or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of -110 W. Modelling approaches illustrate the link between protonmotive force and currents (Willis et al. 2016).

We consider isolated mitochondria as powerhouse and proton pumps as molecular machines to relate experimental results to energy metabolism of the intact cell. The cellular $P_s/O_2$ based on oxidation...
of glycogen is increased by the glycolytic (fermentative) substrate-level phosphorylation of 3 P»/Glyc or 0.5 mol P» for each mol O2 consumed in the complete oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial P»/O2 ratio of 5.4 yields a bioenergetic cell physiological P»/O2 ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either by the malate-aspartate shuttle or by the glycerophosphate shuttle (Figure 2A) resulting in different theoretical yields 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»/O2 ratio not only reflects proton translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger 1993a).

7. Conclusions

Catabolic cell respiration is the process of exergonic and 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. An O2 flux balance scheme illustrates the relationships and general definitions (Figures 1 and 2).

Box 3: Recommendations for studies with mitochondrial preparations

- Normalization of respiratory rates should be provided as far as possible:
  1. Biophysical normalization: on a per cell basis as O2 flow; this may not be possible when dealing with coenocytic organisms or tissues without cross-walls separating individual cells (e.g., filamentous fungi, muscle fibers)
  2. Cellular normalization: per g protein; per cell- or tissue-mass as mass-specific O2 flux; per cell volume as cell volume-specific flux
  3. Mitochondrial normalization: per mitochondrial marker as mt-specific flux.

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). Reporting flow in a respiratory chamber [nmol∙s⁻¹] is discouraged, since it restricts the analysis to intra-experimental comparison of relative (qualitative) differences.

- Catabolic mitochondrial respiration is distinguished from residual O2 consumption. Fluxes in mitochondrial coupling states should be, as far as possible, corrected for residual O2 consumption.

- Different mechanisms of uncoupling should be distinguished by defined terms. The tightness of coupling relates to these uncoupling mechanisms, whereas the coupling stoichiometry varies as a function the substrate type involved in ET-pathways with either three or two redox proton pumps operating in series. Separation of tightness of coupling from the pathway-dependent coupling stoichiometry is possible only when the substrate type undergoing oxidation remains the same for respiration in LEAK-, OXPHOS-, and ET-states. In studies of the tightness of coupling, therefore, simple substrate-inhibitor combinations should be applied to exclude a shift in substrate competition which may occur when providing physiological substrate cocktails.

- In studies of isolated mitochondria, the mitochondrial recovery and yield should be reported. Experimental criteria for evaluation of purity versus integrity should be considered. 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 recovery, i.e., the fraction of mitochondrial marker obtained from a unit mass of tissue. Total mitochondrial protein is frequently applied as a mitochondrial marker, which is restricted to isolated mitochondria.

- In studies of permeabilized cells, the viability of the cell culture or cell suspension of origin should be reported. Normalization should be evaluated for total cell count or viable cell count.

- Terms and symbols are summarized in Table 8. Their use will facilitate transdisciplinary communication and support further developments towards a consistent theory of bioenergetics and mitochondrial physiology. Technical terms related to and defined with normal words can be used as index terms in databases, support the creation of ontologies towards semantic information processing
Making data available without making it understandable may be worse than not making it available at all (National Academies of Sciences, Engineering, and Medicine 2018). Success will depend on taking next steps: (1) exhaustive text-mining considering Omics data and functional data; (2) network analysis of Omics data with bioinformatics tools; (3) cross-validation with distinct bioinformatics approaches; (4) correlation with functional data; (5) guidelines for biological validation of network data. This is a call to carefully contribute to FAIR principles (Findable, Accessible, Interoperable, Reusable) for the sharing of scientific data.

Table 8. Terms, symbols, and units.

<table>
<thead>
<tr>
<th>Term</th>
<th>Symbol</th>
<th>Unit</th>
<th>Links and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>alternative quinol oxidase</td>
<td>AOX</td>
<td></td>
<td>Figure 2B</td>
</tr>
<tr>
<td>amount of substance B</td>
<td>( n_B )</td>
<td>[mol]</td>
<td></td>
</tr>
<tr>
<td>ATP yield per ( O_2 )</td>
<td>( Y_{P_o,O_2} )</td>
<td></td>
<td>( P/o_2 ) ratio measured in any respiratory state</td>
</tr>
<tr>
<td>catabolic reaction</td>
<td>( k )</td>
<td>( \text{varies} )</td>
<td>Figure 1 and 3</td>
</tr>
<tr>
<td>catabolic respiration</td>
<td>( J_{i,O_2} )</td>
<td>( \text{varies} )</td>
<td>Figure 1 and 3</td>
</tr>
<tr>
<td>cell number</td>
<td>( N_{ce} )</td>
<td>[x]</td>
<td>( N_{ce} = N_{vce} + N_{dce} ); Table 5</td>
</tr>
<tr>
<td>cell respiration</td>
<td>( J_{r,O_2} )</td>
<td>( \text{varies} )</td>
<td>Figure 1</td>
</tr>
<tr>
<td>cell viability index</td>
<td>( VI )</td>
<td>( \text{varies} )</td>
<td>Table 6; ( \text{O}_2 = 4 )</td>
</tr>
<tr>
<td>Complexes I to IV</td>
<td>CI to CIV</td>
<td></td>
<td>respiratory ET Complexes; Figure 2B</td>
</tr>
<tr>
<td>concentration of substance B</td>
<td>( c_B = n_B \cdot V^{-1}; ) ([B]) ([\text{mol} \cdot \text{m}^{-3}])</td>
<td>Box 2</td>
<td></td>
</tr>
<tr>
<td>dead cell number</td>
<td>( N_{dce} )</td>
<td>[x]</td>
<td>non-viable cells, loss of plasma membrane barrier function; Table 5</td>
</tr>
<tr>
<td>electric format</td>
<td>( \varepsilon )</td>
<td>[C]</td>
<td>Table 6</td>
</tr>
<tr>
<td>electron transfer system</td>
<td>ETS</td>
<td></td>
<td>state; Figure 2B, Figure 4</td>
</tr>
<tr>
<td>flow, for substance B</td>
<td>( I_B )</td>
<td>[mol \cdot s^{-1}]</td>
<td>system-related extensive quantity; Figure 6</td>
</tr>
<tr>
<td>flux, for substance B</td>
<td>( J_B )</td>
<td>( \text{varies} )</td>
<td>size-specific quantity; Figure 6</td>
</tr>
<tr>
<td>inorganic phosphate</td>
<td>( P_I )</td>
<td></td>
<td>Figure 2C</td>
</tr>
<tr>
<td>inorganic phosphate carrier</td>
<td>PiC</td>
<td></td>
<td>Figure 2C</td>
</tr>
<tr>
<td>intact cell number</td>
<td>( N_{vce} )</td>
<td>[x]</td>
<td>viable cells, intact of plasma membrane barrier function; Table 5</td>
</tr>
<tr>
<td>LEAK state</td>
<td>LEAK</td>
<td></td>
<td>state; Table 1, Figure 4</td>
</tr>
<tr>
<td>mass format</td>
<td>( m )</td>
<td>[kg]</td>
<td>Table 4</td>
</tr>
<tr>
<td>mass of sample ( X )</td>
<td>( m_X )</td>
<td>[kg]</td>
<td>Table 4</td>
</tr>
<tr>
<td>mass, dry mass</td>
<td>( m_d )</td>
<td>[kg]</td>
<td>mass of sample ( X ); Figure 6</td>
</tr>
<tr>
<td>mass, wet mass</td>
<td>( m_w )</td>
<td>[kg]</td>
<td>mass of sample ( X ); Figure 6</td>
</tr>
<tr>
<td>mass of object ( X )</td>
<td>( M_X = m_X \cdot N_X^{-1} )</td>
<td>[kg \cdot x^{-1}]</td>
<td>mass of entity ( X ); Table 4</td>
</tr>
</tbody>
</table>
Experimentially, respiration is separated in mitochondrial preparations from the interactions with the fermentative pathways of the intact cell. OXPHOS analysis is based on the study of mitochondrial preparations complementary to bioenergetic investigations of intact cells and organisms—from model organisms to the human species including healthy and diseased persons (patients). Different mechanisms of respiratory uncoupling have to be distinguished (Figure 3). Metabolic fluxes measured in defined...
coupling and pathway control states (Figures 5 and 6) provide insights into the meaning of cellular and organismic respiration.

The optimal choice for expressing mitochondrial and cell respiration as O\textsubscript{2} flow per biological sample, 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 under study. Interpretation of the data depends critically on appropriate normalization (Figure 6).

MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory adaptations and 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, are focused on studies with mitochondrial preparations (Box 3). These will be extended in a series of reports on pathway control of mitochondrial respiration, respiratory states in intact cells, and harmonization of experimental procedures.

Acknowledgements

We thank M. Beno for management assistance. This publication is based upon work from COST Action CA15203 MitoEAGLE, supported by COST (European Cooperation in Science and Technology), and K-Regio project MitoFit (E.G.).

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

References


volume density by enlargement of existing mitochondria and not de novo biogenesis. Acta Physiol 222, e12905.


Supplement

S1. Manuscript phases and versions - an open-access approach

This manuscript on ‘Mitochondrial respiratory states and rates’ is a position statement in the frame of COST Action CA15203 MitoEAGLE. The list of co-authors evolved beyond phase 1 in the bottom-up spirit of COST.

The global MitoEAGLE network made it possible to collaborate with a large number of co-authors to reach consensus on the present manuscript. Nevertheless, we do not consider scientific progress to be supported by ‘declaration’ statements (other than on ethical or political issues). Our manuscript aims at providing arguments for further debate rather than pushing opinions. We hope to initiate a much broader process of discussion and want to raise the awareness on the importance of a consistent terminology for reporting of scientific data in the field of bioenergetics, mitochondrial physiology and pathology. Quality of research requires quality of communication.

Some established researchers in the field may not want to re-consider the use of jargon which has become established despite deficiencies of accuracy and meaning. In the long run, superior standards will become accepted.

We hope to contribute to this evolutionary process, with an emphasis on harmonization rather than standardization.

Phase 1 The protonmotive force and respiratory control

- 2017-04-09 to 2017-09-18 (44 versions)
- 2017-09-21 to 2018-02-06 (44+21 versions)

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

Phase 2 Mitochondrial respiratory states and rates: Building blocks of mitochondrial physiology Part 1

http://www.mitoeagle.org/index.php/MitoEAGLE_preprint_2017-09-21

2017-11-11: Print version (16) for MiP2017/MitoEAGLE conference in Hradec Kralove

Phase 3 Submission to a preprint server: BioRxiv

Phase 4 Journal submission

CELL METABOLISM, aiming at indexing by The Web of Science and PubMed.

S2. Authors

This manuscript developed as an open invitation to scientists and students to join as co-authors, to provide a balanced view on mitochondrial respiratory control and a consensus statement on reporting data of mitochondrial respiration in terms of metabolic flows and fluxes.

Co-authors are added in alphabetical order based upon a first draft written by the corresponding author, who edited all versions. Co-authors confirm to have read the final manuscript, possibly have made additions or suggestions for improvement, and agree to implement the recommendations into future manuscripts, presentations and teaching materials.

We continue to invite comments and suggestions, 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, providing direct input and references, and 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, we 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.

S3. Joining COST Actions

- CA15203 MitoEAGLE - http://www.cost.eu/COST_Actions/ca/CA15203
- CA16225 EU-CARDIOPROTECTION - http://www.cost.eu/COST_Actions/ca/CA16225
- CA17129 CardioRNA - http://www.cost.eu/COST_Actions/ca/CA17129
Mitochondrial respiratory states and rates: Building blocks of mitochondrial physiology

Aims
Clarity of concept and consistency of nomenclature facilitate effective transdisciplinary communication, education, and ultimately further discovery.

Adhering to uniform standards and harmonizing the terminology concerning mitochondrial respiratory states and rates will support the development of databases of mitochondrial respiratory function in cells, tissues, and species.

Summary
Recommendations on coupling control states and rates are focused on studies with mitochondrial preparations.

Fig. 1: Respiration is defined by O2 flux balance.

Fig. 2: OXPHOS analysis is based on the study of mt-preparations. Metabolic fluxes measured in defined coupling and pathway control states provide insights into the meaning of cellular respiration.

Fig. 3: Interpretation of respiratory rates depends critically on appropriate normalization.

Table 1. Coupling states and residual oxygen consumption in mitochondrial preparations in relation to respiration- and phosphorylation-flux, \( \Delta p \), and \( \Delta m \).

<table>
<thead>
<tr>
<th>State</th>
<th>( \Delta p )</th>
<th>( \Delta m )</th>
<th>Indicating Factors</th>
<th>Limiting Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEAK</td>
<td>Low, cell-</td>
<td>Low, cell-</td>
<td>min leak, slip, and cation cycling,</td>
<td>ATP-ADP ratio; ADR; or (4) inhibition of the phosphorylation-pathway, ( \Delta m ).</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>High, ADP-</td>
<td>Max, high</td>
<td>kinetically-saturating [ADP] and [Pi]</td>
<td>ATP-ADP ratio; ADR; or (4) inhibition of the phosphorylation-pathway, ( \Delta m ).</td>
</tr>
<tr>
<td>ET</td>
<td>Max, uncoupled respiration</td>
<td>Low optimal external uncoupler concentration for max. ( \Delta m )</td>
<td>( \Delta m ) by phosphorylation-pathway, or ( \Delta m ) by ET-capacity</td>
<td>( \Delta m ) by ET-capacity</td>
</tr>
<tr>
<td>ROX</td>
<td>ROX, min. residual ( O_2 ) consumption</td>
<td>0</td>
<td>( \Delta m ) by non-ET-pathway oxidation reactions, ( \Delta m ) by non-ET-pathways, or rate of fuel substrates</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. From mitochondrial to external respiration
Mitochondrial (mt) respiration is the oxidation of fuel substrates (electron donors) and reduction of \( O_2 \) catalyzed by the electron transfer system, ET.

Figure 2. Respiratory states (ET, OXPHOS, LEAK) and corresponding rates (E, P, L).

Figure 3. Normalization of rate
A: Cell respiration is normalized for (1) the experimental sample (flow per object, mass-specific flux, or cell-volume-specific flux); or (2) for methodological reasons for the Chamber volume.

B: Flow per cell (mol \( O_2 \) s\(^{-1}\) cell\(^{-1}\)) is flux per chamber volume, \( \Delta m \) (mol \( O_2 \) s\(^{-1}\) L\(^{-1}\)), divided by cell concentration in the chamber, \( \Delta m \) (mol \( O_2 \) s\(^{-1}\) L\(^{-1}\)), which is Number analysis. In Structure analysis, aerobic cell performance is mt-quality (mt-specific flux, e.g., per citrate synthase, CS) times mt quantity, or mt-function times mt-structure.
Mission of the global MitoEAGLE network
in collaboration with the Mitochondrial Physiology Society, MiPs

- Improve our knowledge on mitochondrial function in health and disease with regard to Evolution, Age, Gender, Lifestyle and Environment
- Interrelate studies across laboratories with the help of a MitoEAGLE data management system
- Provide standardized measures to link mitochondrial and physiological performance to understand the myriad of factors that play a role in mitochondrial physiology

Join the COST Action MitoEAGLE - contribute to the quality management network.

More information: www.mitoeagle.org

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