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Consortium communication

Mitochondrial physiology
2. Respiratory states and rates

MitoEAGLE Task Group*

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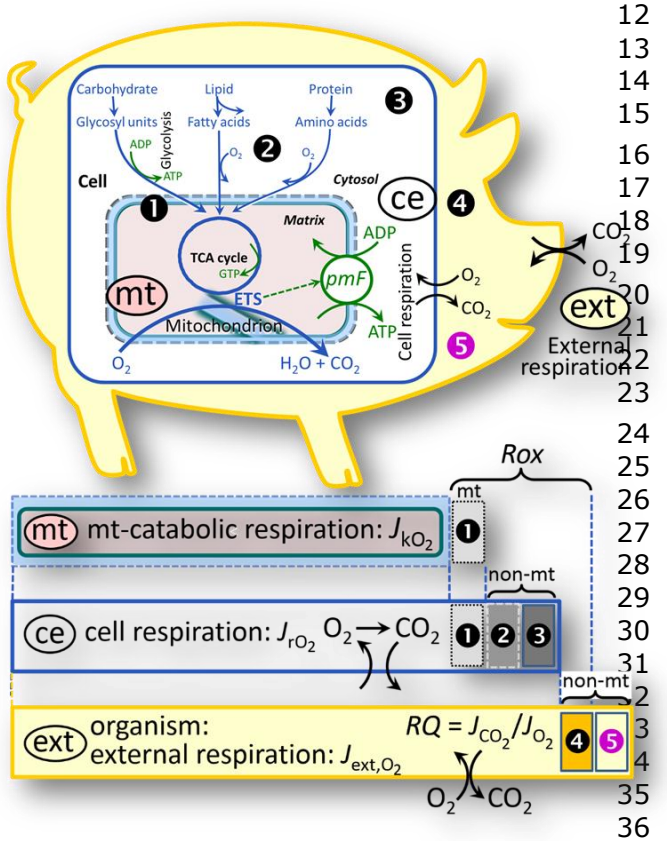

Overview

Internal and external respiration

(mt) **Mitochondrial catabolic respiration**, J_{kO_2}, is the O_2 consumption in the oxidation of fuel substrates (electron donors) and reduction of O_2 catalysed by the electron transfer system, ETS, which drives the protonmotive force, *pmF*. J_{kO_2} excludes mitochondrial residual oxygen consumption, mt-*Rox* (❶).

(ce) **Cell respiration** or internal cellular O_2 consumption, J_{rO_2}, takes into account all chemical reactions, r, that consume O_2 in the cells. Catabolic cell respiration is the O_2 consumption associated with catabolic pathways in the cell, including (mt) mitochondrial catabolism; mt-*Rox* (❶); non-mt O_2 consumption by catabolic reactions, particularly peroxisomal oxidases and microsomal cytochrome P450 systems (❷); and non-mt *Rox* by reactions unrelated to catabolism (❸).

(ext) **External respiration** balances internal respiration at steady-state, including extracellular *Rox* (❹) and aerobic respiration by the microbiome (❺).

O_2 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 CO_2/O_2 exchange ratio; when combined with the respiratory nitrogen quotient, N/O_2 (mol N given off per mol O_2 consumed), the *RQ* reflects the proportion of carbohydrate, lipid and protein utilized in cell respiration during aerobically balanced steady-states. Bicarbonate and CO_2 are transported in reverse to the extracellular milieu and the organismic environment. Hemoglobin provides the molecular paradigm for the combination of O_2 and CO_2 exchange, as do lungs, gills, the skin and other surfaces on the morphological level.

Respiratory states are defined in **Table 1**. Rates are illustrated in **Figure 5**. Consult **Table 8** for a list of terms and symbols.

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Table and Figure numbers refer to Gnaiger *et al* (2020) Bioenerg Commun 2020.1.

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Abstract

As the knowledge base and importance of mitochondrial physiology to evolution, health and disease 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 guidelines of the International Union of Pure and Applied Chemistry (IUPAC) on terminology in physical chemistry, extended by considerations of open systems and thermodynamics of irreversible processes. The concept-driven constructive terminology incorporates the meaning of each quantity and aligns concepts

and symbols with the nomenclature of classical bioenergetics. We endeavour to provide a balanced view of 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, ion leak and slip compensatory state: LEAK, residual oxygen consumption: ROX, State 2, State 3, State 4, normalization, flow, flux, oxygen: O_2

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 is the O_2 flux in catabolic reactions coupled to phosphorylation of ADP to ATP, and O_2 flux in a variety of O_2 consuming reactions apart from oxidative phosphorylation (OXPHOS). Coupling in OXPHOS is mediated by the translocation of protons across the mitochondrial inner membrane (mtIM) 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 depends on ion translocation across a semipermeable membrane, which is defined as vectorial metabolism and distinguishes OXPHOS from cytosolic fermentation as counterparts of cellular core energy metabolism (**Overview**). Cell respiration is thus 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 O_2 as the electron acceptor. (*2*) Compartmental coupling in vectorial OXPHOS 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, extramitochondrial 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, *pmF*, in an interaction of the electron transfer system generating the *pmF* and the phosphorylation system utilizing the *pmF*. Capacities of OXPHOS and electron transfer are measured at kinetically-saturating concentrations of fuel substrates, ADP and inorganic phosphate, and O_2, or at optimal uncoupler concentrations, respectively, in the absence of Complex IV inhibitors such as NO, CO, or H_2S. Respiratory capacity is a measure of the upper

boundary of the rate of respiration; it depends on the substrate type undergoing oxidation in a mitochondrial pathway, and provides reference values for the diagnosis of health and disease. Evaluation of the impact of evolutionary background, age, gender and sex, lifestyle and environment represents a major challenge for mitochondrial respiratory physiology and pathology.

4. Incomplete tightness of coupling, *i.e.*, some degree of uncoupling relative to the mitochondrial pathway-dependent coupling 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 instrumental chamber are sample-specific normalizations, which are distinguished from system-specific normalization for the volume of the instrumental chamber (the measuring system).

6. The consistent use of terms and symbols facilitates transdisciplinary communication and will support the further development of a collaborative database on bioenergetics and mitochondrial physiology.

1. Part 2. States and rates

Mitochondria are the powerhouses of the cell with numerous morphological, physiological, molecular, and genetic functions. Every study of mitochondrial health and disease faces **E**volution, **A**ge, **G**ender and sex, **L**ifestyle, and **E**nvironment (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 depend on accurate measurements under strictly-defined conditions. Likewise, meaningful interpretation and comparability of experimental outcomes requires harmonization of protocols between research groups at different institutes. In addition to quality control, a conceptual framework is also required to standardise and harmonise terminology and methodology. Vague or ambiguous jargon can lead to confusion and may convert 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 data repositories 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 in the attempt to establish a conceptually-oriented nomenclature in bioenergetics and mitochondrial physiology in a series of communications, prepared in the frame of the EU COST Action MitoEAGLE open to global bottom-up input. Reference to Part 1. Quantitative mitochondrial physiology. Mitochondria and bioblasts

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 (Diebold *et al* 2019). Glycolysis involves substrate-level phosphorylation of ADP to ATP in fermentation without utilization of O_2, studied mainly in living 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 $FADH_2$. Subsequent mitochondrial electron transfer to O_2 is coupled to proton translocation for the control of the protonmotive force and phosphorylation of ADP (**Figure 1b and 1c**). In contrast,extramitochondrial oxidation of fatty acids and amino acids proceeds partially in peroxisomes without coupling to ATP production: acyl-CoA oxidase catalyzes the oxidation of $FADH_2$ with electron transfer to O_2; amino acid oxidases oxidize flavin mononucleotide $FMNH_2$ or $FADH_2$ (**Figure 1a**).

The plasma membrane separates the intracellular compartment including the cytosol, nucleus, and organelles from the extracellular environment. Cell membranes include the plasma membrane and organellar membranes. 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 (P_i) that must be precisely controlled at kinetically-saturating concentrations for the analysis of mitochondrial respiratory capacities (**Figure 2**). Respiratory capacities delineate, comparable to channel capacity in information theory (Schneider 2006), the upper boundary of the rate of O_2 consumption measured in defined respiratory states. The intact plasma membrane limits the scope of investigations into mitochondrial respiratory function in living cells, despite the activity of solute carriers, *e.g.*, the sodium-dependent dicarboxylate transporter SLC13A3 and the sodium-dependent phosphate transporter SLC20A2, which transport specific metabolites across the plasma membrane of various cell types, and the availability of plasma membrane-permeable succinate (Ehinger *et al* 2016). These limitations are overcome by the use of mitochondrial preparations.

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.

Kinetically-saturating conditions are evaluated by substrate kinetics to obtain the maximum reaction velocity or maximum pathway flux, in contrast to solubility-saturated conditions. When aiming at the measurement of kinetically-saturated processes—such as OXPHOS-capacities—the concentrations for substrates can be chosen according to half-saturating substrate concentrations, c_{50}, for metabolic pathways, or the Michaelis constant, K_m, for enzyme kinetics. In the case of hyperbolic kinetics, only 80 % of maximum respiratory capacity is obtained at a substrate concentration of four times the c_{50}, whereas substrate concentrations of 5, 9, 19 and 49 times the c_{50} 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 safranin, 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 (O_2; 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 [O_2] 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·10^{-6} cells) is optimal for permeabilization of endothelial cells, and the concentration in the incubation medium has to be adjusted according to the cell concentration (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* neither includes living cells, nor submitochondrial particles and further fractionated 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 1b**). 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 CII to fumarate. CII is part of both the TCA cycle and the ETS, and reduces FAD to $FADH_2$ with further reduction of ubiquinone to ubiquinol downstream of the TCA cycle in the Q-junction. Thus $FADH_2$ is not a substrate but is the product of CII, in contrast to erroneous metabolic maps shown in many publications. β-oxidation of fatty acids (FA) supplies reducing equivalents via (*1*) $FADH_2$ as the substrate of electron transferring flavoprotein complex (CETF); (*2*) acetyl-CoA generated by chain shortening; and (*3*) NADH generated via 3-hydroxyacyl-CoA dehydrogenases. The ATP yield depends on whether acetyl-CoA enters the TCA cycle, or is for example used in ketogenesis.

Selected mitochondrial catabolic pathways of electron transfer from the oxidation of fuel substrates to the reduction of O_2 are stimulated by addition of fuel substrates to the mitochondrial respiration medium after depletion of endogenous substrates (**Figure 1b**). Substrate combinations and specific inhibitors of ET-pathway enzymes are used to obtain defined pathway control states in mitochondrial preparations (Gnaiger 2020).

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 O_2 flux, J_{kO_2} (**Figure 1**). 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 change. Exergy is not completely conserved, however, except at the limit of 100 % efficiency of energy transformation in a coupled process. The exergy or Gibbs energy change that is not conserved by copling is irreversibly lost or dissipated, and is accounted for as the entropy change of the surroundings and the system, multiplied by the temperature of the irreversible 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 1**).

2.4.2. Phosphorylation, P», and P»/O_2 ratio: Phosphorylation in the context of OXPHOS is defined as phosphorylation of ADP by P_i to form ATP. On the other hand, the term phosphorylation is used generally in many contexts, *e.g.,* protein phosphorylation. This provides the argument for introducing 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 1**): The symbol P» indicates the endergonic (uphill) direction of phosphorylation ADP→ATP, and likewise P« 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 (Komlódi and Tretter 2017). Kinase cycles are involved in intracellular energy transfer and signal transduction for regulation of energy flux.The P»/O_2 ratio (P»/4 e^-) is two times the 'P/O' ratio (P»/2 e^-). P»/O_2 is a generalized symbol, not specific for reporting P_i consumption (P_i/O_2 flux ratio), ADP depletion (ADP/O_2 flux ratio), or ATP production (ATP/O_2 flux ratio). The mechanistic P»/O_2 ratio—or P»/O_2 stoichiometry—is calculated from the proton–to–O_2 and proton–to–phosphorylation coupling stoichiometries (**Figure 1c**):

$$P»/O_2 = \frac{H^+_{pos}/O_2}{H^+_{neg}/P»} \qquad (1)$$

The H^+_{pos}/O_2 coupling stoichiometry (referring to the full four electron reduction of O_2) 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 O_2 (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. AOX are expressed in all plants, some fungi, many protists, and several animal phyla, but are not expressed in vertebrate mitochondria (McDonald *et al* 2009).

The H^+_{pos}/O_2 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 1b**). A general consensus on H^+_{pos}/O_2 stoichiometries, however, remains to be reached (Hinkle 2005; Wikström and Hummer 2012; Sazanov 2015). The H^+_{neg}/P» coupling stoichiometry (3.7; **Figure 1b**) is the sum of 2.7 H^+_{neg} required by the F_1F_O-ATPase of vertebrate and most invertebrate species (Watt *et al* 2010) and the proton balance in the translocation of ADP, ATP and P_i (**Figure 1c**). Taken together, the mechanistic P»/O_2 ratio is calculated at 5.4 and 3.3 for the N- and S-pathway, respectively (Eq. 1). The corresponding classical P»/O ratios (referring to the 2 electron reduction of 0.5 O_2) are 2.7 and 1.6 (Watt *et al* 2010), in agreement with the measured P»/O ratio for succinate of 1.58 ± 0.02 (Gnaiger *et al* 2000).

2.4.3. Uncoupling: The effective P»/O_2 flux ratio ($Y_{P»/O_2} = J_{P»}/J_{kO_2}$) is diminished relative to the mechanistic P»/O_2 ratio by intrinsic and extrinsic uncoupling or dyscoupling (**Figure 3**). This is distinct from switching between 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 bypassing CIII and CIV through AOX (**Figure 1b**). 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, $Y_{P»/O_2}$ 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 (**Figure 3**):

1. Proton leak across the mtIM from the positive to the negative compartment (H^+ leak-uncoupled);
2. Cycling of other cations, strongly stimulated by mtPT; 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 (CI, CIII and CIV) when protons are effectively not pumped in the ETS, or are not driving phosphorylation (F_1F_0-ATPase)
4. Loss of vesicular (compartmental) integrity when electron transfer is acoupled
5. Electron leak in the loosely coupled univalent reduction of O_2 to superoxide ($O_2^{\bullet-}$; superoxide anion radical)

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

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). Diagnostically meaningful and reproducible conditions are defined for measuring mitochondrial function and respiratory capacities of core energy metabolism. Standard respiratory coupling states are obtained while maintaining a defined ET-pathway state with constant fuel substrates and inhibitors of specific branches of the ET-pathway. 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**).

LEAK: 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.

OXPHOS: The ET- and phosphorylation-pathways comprise coupled segments of the OXPHOS-system and provide reference values of respiratory capacities. The OXPHOS-capacity is measured at kinetically-saturating concentrations of ADP and P_i.

ET: Compared to OXPHOS-capacity, the oxidative ET-capacity reveals the limitation of OXPHOS-capacity mediated by the phosphorylation-pathway. By application of external uncouplers, ET-capacity is measured as noncoupled respirationThe three coupling states, LEAK, OXPHOS, and ET are shown schematically with the corresponding respiratory rates, abbreviated as *L*, *P*, and *E*, respectively (**Figure 2**). We distinguish between metabolic *pathways* and metabolic *states* with the corresponding metabolic *rates*; for example: ET-pathways, ET-states, and ET-capacities, *E*, respectively (**Table 1**). The protonmotive force, *pmF*, is *maximum* in the LEAK-state of coupled mitochondria, driven by LEAK-respiration at a minimum back-flux of cations to the matrix side, *high* in the OXPHOS-state when it drives phosphorylation, and *very low* in the ET-state when uncouplers short-circuit the proton cycle (**Table 1**).

2.5.1. LEAK-state (**Figure 4a**): 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_i. 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_1F_0-ATPase (oligomycin), or adenine nucleotide translocase (carboxyatractyloside). Adjustment of

the nominal concentration of these inhibitors to the concentration of biological sample applied can minimize or avoid inhibitory side-effects exerted on ET-capacity or even some dyscoupling. The chelator EGTA is added to mt-respiration media to bind free Ca^{2+}, thus limiting cation cycling. The LEAK-rate is a function of respiratory state, hence it depends on the (*1*) barrier function of the mtIM ('leakiness'), (*2*) electrochemical potential differences and concentration differences across the mtIM, and (*3*) H^+/O_2 ratio of the ET-pathway (**Figure 1b**).

- **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 4a**). 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 contamination 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 (Jezek *et al* 2018). Consequently, this short-circuit lowers the *pmF* 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_1F_0-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»/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.
- **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 mtPT pore. Dyscoupled respiration is distinguished from experimentally induced *noncoupled* respiration in the ET-state (**Table 2**).
- **Protonophore titration and noncoupled respiration:** Protonophores are uncouplers which are titrated to obtain maximum *noncoupled* respiration as a measure of ET-capacity.
- **Loss of compartmental integrity and acoupled respiration:** Electron transfer and catabolic O_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 acoupled respiration, which is a nonvectorial dissipative process without control by the protonmotive force.

2.5.2. OXPHOS-state (**Figure 4b**): The OXPHOS-state is defined as the respiratory state with kinetically-saturating concentrations of O_2, respiratory and phosphorylation substrates, and absence of exogenous uncoupler, which provides an estimate of the maximal 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 kinetically-saturate flux in isolated mitochondria (Gnaiger 2001; Puchowicz *et al* 2004); greater [ADP] is required, particularly in permeabilized muscle fibers and cardiomyocytes, to overcome limitations by intracellular diffusion and by the reduced conductance of the mtOM (Jepihhina *et al* 2011; Illaste *et al* 2012; Simson *et al* 2016), either through interaction with tubulin (Rostovtseva *et al* 2008) or other intracellular structures (Birkedal *et al* 2014). In addition, kinetically-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 fiber bundles of high respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks *et al* 1998), consistent with experimental evidence that >90 % kinetic 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). 2.5 to 5 mM ADP is sufficient to obtain the actual OXPHOS-capacity in many types of permeabilized tissue and cell preparations, but experimental validation is required in each specific case.

2.5.3. Electron transfer-state (Figure 4c): O_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_2, respiratory substrate and optimum exogenous uncoupler concentration for maximum O_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 $J_{P»} = 0$. The most frequently used uncouplers are carbonyl cyanide m-chloro phenyl hydrazone (CCCP), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), or dinitrophenol (DNP). Stepwise titration of uncouplers stimulates respiration up to or above the level of O_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. There is a need for new protonophoric uncouplers that drive maximal respiration across a broad dosing range and do not inhibit respiration at high concentrations (Kenwood *et al* 2013). The effect on ET-capacity of the reversed function of F_1F_O-ATPase ($J_{P«}$; **Figure 4c**) can be evaluated in the presence and absence of extramitochondrial ATP.

2.5.4. ROX state: The state of residual O_2 consumption, ROX, is not a coupling state, but is relevant to assess respiratory function (**Overview**). The rate of residual oxygen consumption, *Rox*, is defined as O_2 consumption due to oxidative reactions measured after inhibition of ET with rotenone, 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 used to correct respiration measured in defined coupling control states. *Rox*-corrected *L*, *P* and *E* are not only lower than total fluxes, but also change the flux control ratios *L*/*P* and *L*/*E*. *Rox* is not necessarily equivalent to non-mitochondrial reduction of O_2, considering O_2-consuming reactions in mitochondria that are not related to ET—such as O_2 consumption in reactions catalyzed by monoamine oxidases (type A and B), monooxygenases (cytochrome P450 monooxygenases), dioxygenases (trimethyllysine dioxygenase), and several hydoxylases. Isolated mitochondrial fractions, especially those obtained from liver, may be contaminated by peroxisomes, as shown by transmission electron microscopy. This fact makes the exact determination of mitochondrial O_2 consumption and mitochondria-associated generation of reactive oxygen species complicated (Schönfeld *et al* 2009; Speijer 2016; **Figure 1**). The variability of ROX-linked O_2 consumption needs to be studied in relation to non-ET enzyme activities, availability of specific substrates, O_2 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 ET-excess capacity pushing the phosphorylation-flux to the limit of its capacity for utilizing the protonmotive force (**Figure 2**). 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* (Lemieux *et al* 2011). The ET-excess 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. 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 ET-excess capacity is overestimated if kinetically non-saturating [ADP] or [P_i] are used. See State 3 in the next section.

The net OXPHOS-capacity is calculated by subtracting *L* from *P* (**Figure 2**). The net P»/O_2 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 O_2 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 2**).

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 1b**). When substrate cocktails are used supporting the convergent NADH- and succinate-pathways simultaneously, 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 2020). Under these experimental conditions, we cannot separate the tightness of coupling *versus* coupling stoichiometry as the mechanisms of respiratory control in a shift of *L*/*P* ratios. The tightness of coupling and fully coupled O_2 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 1b**).

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.*, O_2 consumption, are instantaneously compensated for by external fluxes across the system boundary, *e.g.*, O_2 supply, preventing a change of O_2 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 O_2, fuel substrates, ADP, P_i, H^+) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require respiratory media with sufficient buffering

capacity and 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 distinguished in this nomenclature.

2.6.1. State 1 is obtained after addition of isolated mitochondria to air-saturated isoosmotic/isotonic respiration medium containing P_i, but no fuel substrates and no adenylates.

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 O_2 flux, State 2 is equivalent to the ROX state (**Table 1**). Undefined endogenous fuel substrates are a confounding factor of pathway control, contributing to the effect of subsequently externally added substrates and inhibitors. In an alternative sequence of titration steps, the second state is induced by addition of fuel substrate without ADP or ATP (Estabrook 1967). In contrast to the original State 2 defined in **Table 1** as a ROX state, the alternative 'State 2' is a LEAK-state with L(n). Some researchers have called this condition as 'pseudostate 4'.

2.6.3. State 3 is the state stimulated by addition of fuel substrates while the ADP concentration in the original State 2 is still high (**Table 3**) and supports coupled energy transformation. '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 O_2 concentrations near air-saturation (193 or 238 µM O_2 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 O_2 flux that does not lead to O_2 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 O_2 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 $Y_{P»/O_2}$ towards diminishing [ADP] at State 4 must be taken into account for calculation of P»/O_2 ratios (Gnaiger 2001). State 4 respiration, L(T) (**Table 1**), reflects intrinsic proton leak and ATP hydrolysis activity. O_2 flux in State 4 is an overestimation of LEAK-respiration if the contaminating ATP hydrolysis activity recycles some ATP to ADP, $J_{P«}$, which stimulates respiration coupled to phosphorylation, $J_{P»} > 0$. Some degree of mechanical disruption and loss of mitochondrial integrity allows the exposed mitochondrial F_1F_0-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 $J_{P»} = 0$ (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 O_2 is available. Anoxia may be reached, however, before exhaustion of ADP (State 5).

2.6.5. State 5 '*may be obtained by antimycin A treatment or by anaerobiosis*' (Chance and Williams, 1955). These definitions give State 5 two different meanings: ROX or anoxia. Anoxia is obtained after exhaustion of O_2 in a closed respirometric chamber. Diffusion of O_2 from the surroundings into the aqueous solution may be a confounding factor preventing complete anoxia (Gnaiger 2001).

In **Table 3**, only States 3 and 4 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

Reference to Part 1. Quantitative mitochondrial physiology. Mitochondria and bioblasts

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 data repository. 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*, I, when expressed per *object* (per number of cells or organisms) or per chamber (per instrumental 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 5A; Box 1**).

Box 1: Metabolic flows and fluxes: vectoral, vectorial, and scalar

Flow is an extensive quantity (I; per system), distinguished from flux as a size-specific quantity (J; per system size). *Flows,* I_{tr}, are defined for all transformations as extensive quantities. This is a generalization derived from electrical terms: Electric charge per unit time is electric flow or current, $I_{el} = dQ_{el} \cdot dt^{-1}$ [A≡ C·s^{-1}]. When dividing I_{el} by size of the system (cross-sectional area of a 'wire'), we obtain flux as a size-specific quantity; this is the current density (surface-density of flow) perpendicular to the direction of flux, $\boldsymbol{J}_{el} = I_{el} \cdot A^{-1}$ [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 $\boldsymbol{J}_{tr} = I_{tr} \cdot A^{-1}$ [mol·s^{-1}·m^{-2}] and $J_{tr} = I_{tr} \cdot V^{-1}$ [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 defining: (*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 localized transformations without translocation, such as chemical reactions in a homogenous system (catabolic O_2 flux, J_{kO_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). The term *specific* has different meanings in three particular contexts: (*1*) In the system-paradigm, (*a*) mass-specific flux is flow divided by mass of the system (the mass of everything contained in the instrumental chamber or reactor). (*b*) Rates are frequently expressed as volume-specific flux (volume of the instrumental chamber). A mass-specific or volume-specific quantity is independent of the extent of non-interacting homogenous subsystems. (*2*) In the context of *sample size*, tissue-specific quantities are related to the mass or volume of the sample in contrast to the mass or volume of the *system* (*e.g.*, muscle mass-specific or cell volume-specific normalization; **Figure 5**). (*3*) An entirely different meaning is implied in the context of *sample type* (*e.g.*, muscle-specific compared to brain-specific properties).
- **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).
- **Formats:** The quantity of a sample *X* can be expressed in different formats. n_X, N_X, and m_X are the molar amount, number, and mass of *X*, respectively. When different formats are indicated in symbols of derived quantities, the format ($\underline{n}$, $\underline{N}$, $\underline{m}$) is shown as a subscript (*underlined italic*), such as in $I_{O_2/\underline{N}X}$ and $J_{O_2/\underline{m}X}$. As of 2019 May 20, the definition of the SI unit mole [mol] is based on a natural constant, namely Avogadro's constant: one mole contains exactly $6.02214076 \cdot 10^{23}$ elementary entities, in contrast to the former definition in terms of the number of atoms in the mass of 0.012 kilogram of carbon 12 (Gibney 2018). Metabolic 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. These formats are distinguished as $J_{\underline{n}O_2/\underline{m}X}$ and $J_{\underline{V}O_2/\underline{m}X}$, respectively, for mass-specific flux. Further examples are given in **Table 4** and **Figure 5**.

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 5**).

4.1. Flow: per object

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

4.1.2. Flow per object, $I_{O_2/\underline{N}X}$: O_2 flow per cell is calculated from volume-specific O_2 flux, J_{V,O_2} [nmol·s^{-1}·L^{-1}] (per *V* of the instrumental 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, $VI = N_{vce} \cdot N_{ce}^{-1}$, is the ratio of the number of viable cells, N_{vce}, per total number of living cells in the population. After experimental permeabilization, all cells are permeabilized, $N_{pce} = N_{ce}$. The cell viability index can be used to normalize respiration for the number of cells that have been viable before experimental permeabilization, $I_{O_2/\underline{N}vce} = I_{O_2/\underline{N}ce} \cdot VI^{-1}$, considering that mitochondrial respiratory dysfunction in dead cells should be eliminated as a confounding factor.

4.2. Size-specific flux: per sample size

4.2.1. Sample concentration, $C_{\underline{m}X}$: 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 mass, m_w or m_d [mg], respectively, or as mass of protein, $m_{Protein}$. The sample concentration is the mass of the subsample per

volume of the instrumental chamber, $C_{mX} = m_X \cdot V^{-1}$ [g·L^{-1} = mg·mL^{-1}]. *X* is the type of sample—isolated mitochondria, tissue homogenate, permeabilized muscle fibers or cells (**Table 4**). m_{ce} [mg] is the total mass of all cells in an instrumental chamber, whereas $m_{Nce} = m_{ce} \cdot N_{ce}^{-1}$ [mg·x^{-1}] is the (average) mass of an individual cell (**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 (Renner *et al* 2003), normalization is required to obtain cell size-specific or mitochondrial marker-specific O_2 flux (**Figure 5**).

- **Mass-specific flux, $J_{O_2/mX}$** [mol·s^{-1}·kg^{-1}]: Mass-specific flux is the expression of respiration per mass of sample, m_X [mg]. Chamber volume-specific flux, J_{V,O_2}, is divided by mass concentration of *X* in the chamber, $J_{O_2/mX} = J_{V,O_2} \cdot C_{mX}^{-1}$. Cell mass-specific flux is obtained by dividing flow per cell by mass per cell, $J_{O_2/mce} = I_{O_2/Nce} \cdot m_{Nce}^{-1}$.
- **Cell volume-specific flux**, $J_{O_2/VX}$ [mol·s^{-1}·m^{-3}]: Sample volume-specific flux is obtained by expressing respiration per volume of sample.

If size-specific O_2 flux is constant and independent of sample size, then there is no interaction between the subsystems. For example, 1.5 mg and 3.0 mg sub-samples of muscle tissue respire at identical mass-specific flux. If mass-specific O_2 flux, however, changes as a function of the mass of a tissue sample, cells or isolated mitochondria in the instrumental chamber, then the nature of the interaction becomes an issue. Therefore, cell concentration 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).

The complexity changes when considering the scaling law of respiratory physiology. Strong interactions are revealed between O_2 flow and body mass of an individual organism: *basal* metabolic rate (flow) does not increase linearly with body mass, whereas *maximum* mass-specific O_2 flux, $\dot{V}_{O2max}$ or $\dot{V}_{O2peak}$, is approximately constant across a large range of individual body mass (Weibel and Hoppeler 2005). Individuals, breeds and species, however, deviate substantially from this relationship. $\dot{V}_{O2peak}$ of human endurance athletes is 60 to 80 mL O_2·min^{-1}·kg^{-1} body mass, converted to $J_{O_2peak/mNorg}$ of 45 to 60 nmol·s^{-1}·g^{-1} (Gnaiger 2020; **Table 6**).

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. 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 suspension of isolated mitochondria, $C_{mtE,stock}$, and crude tissue homogenate, $C_{mtE,thom}$, which together provide information on the specific mitochondrial density in the sample, D_{mtE} (**Table 4**).

When discussing concepts of normalization, it is essential to consider the question posed by 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 in mitochondrial function independent of mitochondrial density (**Table 4**), then normalization to a mitochondrial marker is imperative (**Figure 5**). 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_{mtE}, and mitochondrial markers: Mitochondrial organelles compose 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 structural and functional elementary units of cell respiration*' (Gnaiger 2020). The quantity of a mitochondrial marker can reflect the amount of mitochondrial elementary components, *mtE*, expressed in various mitochondrial elementary units [mtEU] specific for each measured mt-marker (**Table 4**). However, since mitochondrial quality may change in response to stimuli—particularly in mitochondrial dysfunction (Campos *et al* 2017) and after exercise training (Pesta *et al* 2011) and during aging (Daum *et al* 2013)—some markers can vary while others are unchanged: (*1*) Mitochondrial volume and membrane area are structural markers, whereas mitochondrial protein mass is commonly used as a marker for isolated mitochondria. (*2*) Molecular and enzymatic mitochondrial markers (amounts or activities) can be selected as matrix markers, *e.g.*, citrate synthase activity, mtDNA; mtIM-markers, *e.g.*, cytochrome *c* oxidase activity, aa_3 content, cardiolipin, or mtOM-markers, *e.g.*, the voltage-dependent anion channel (VDAC), TOM20. (*3*) Extending the measurement of mitochondrial marker enzyme activity to mitochondrial pathway capacity, ET- or OXPHOS-capacity can be considered as an integrative functional mitochondrial marker.

Depending on the type of mitochondrial marker, the mitochondrial elementary entity, *mtE*, is expressed in marker-specific units. Mitochondrial concentration in the instrumental chamber and mitochondrial density in the tissue of origin are quantified as (*1*) a quantity for normalization in functional analyses, C_{mtE}, and (*2*) a physiological output that is the result of mitochondrial biogenesis and degradation, D_{mtE}, respectively (**Table 4**). It is recommended, therefore, to distinguish *experimental mitochondrial concentration*, $C_{mtE} = mtE \cdot V^{-1}$ and *physiological mitochondrial density*, $D_{mtE} = mtE \cdot m_X^{-1}$. Then mitochondrial density is the amount of mitochondrial elementary components per mass of tissue, which is a biological variable (**Figure 5**). The experimental variable is mitochondrial density multiplied by sample mass concentration in the measuring chamber, $C_{mtE} = D_{mtE} \cdot C_{\underline{m}X}$, or mitochondrial content multiplied by sample number concentration, $C_{mtE} = mtE_X \cdot C_{\underline{N}X}$ (**Table 4**).

4.3.2. mt-Marker-specific flux, $J_{O_2/mtE}$: Volume-specific metabolic O_2 flux depends on: (*1*) the sample concentration in the volume of the instrumental chamber, $C_{\underline{m}X}$, or $C_{\underline{N}X}$; (*2*) the mitochondrial density in the sample, $D_{mtE} = mtE \cdot m_X^{-1}$ or $mtE_X = mtE \cdot N_X^{-1}$; and (*3*) the specific mitochondrial activity or performance per mitochondrial elementary marker, $J_{O_2/mtE} = J_{V,O_2} \cdot C_{mtE}^{-1}$ [mol·s^{-1}·mtEU^{-1}] (**Table 4**). Obviously, the numerical results for $J_{O_2/mtE}$ vary with the type of mitochondrial marker chosen for measurement of *mtE* and $C_{mtE} = mtE \cdot V^{-1}$ [mtEU·m^{-3}].

Reference to Part 3. Quantitative mitochondrial physiology. Mitochondrial markers

Different methods for the quantification of mitochondrial markers have different strengths and weaknesses. Some problems are common for all mitochondrial markers, *mtE*: (*1*) Accuracy of measurement is crucial, since even a highly accurate and reproducible measurement of chamber volume-specific O_2 flux results in an inaccurate and noisy expression if normalized by a biased and noisy measurement of a mitochondrial marker. This problem is acute in mitochondrial respiration because the denominators used (the mitochondrial markers) are often small moieties

of which accurate and precise determination is difficult. In contrast, an *internal* marker is used when O_2 fluxes measured in substrate-uncoupler-inhibitor titration protocols are normalized for flux in a defined respiratory reference state within the assay, which yields flux control ratios, *FCR*s. *FCR*s are independent of externally measured markers and, therefore, are statistically robust, considering the limitations of ratios in general (Jasienski and Bazzaz 1999). *FCR*s indicate qualitative changes of mitochondrial respiratory control, with highest quantitative resolution, separating the effect of mitochondrial density on $J_{O_2/mX}$ and $I_{O_2/NX}$ from that of function per mitochondrial elementary marker, $J_{O_2/mtE}$ (Pesta *et al* 2011; Gnaiger 2020). (*2*) If mitochondrial quality does not change and only the amount of mitochondria varies as a determinant of mass-specific flux, any marker is equally qualified in principle; then in practice selection of the optimum marker depends only on the accuracy and precision of measurement of the mitochondrial marker. (*3*) If mitochondrial flux control ratios change, then there may not be any best mitochondrial marker. In general, measurement of multiple mitochondrial markers enables a comparison and evaluation of normalization for these mitochondrial markers. Particularly during postnatal development, the activity of marker enzymes—such as cytochrome *c* oxidase and citrate synthase—follows different time courses (Drahota *et al* 2004). Evaluation of mitochondrial markers in healthy controls is insufficient for providing guidelines for application in the diagnosis of pathological states and specific treatments.

In line with the concept of the respiratory control ratio (Chance and Williams 1955a), the most readily used normalization is that of flux control ratios and flux control factors (Gnaiger 2020). Selection 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 that indicates stable tissue-mass specific flux.

Stereological measurement of mitochondrial content via two-dimensional transmission electron microscopy is considered as the gold standard in determination of mitochondrial volume fractions in cells and tissues (Weibel, Hoppeler, 2005). Accurate determination of three-dimensional volume by two-dimensional microscopy, however, is 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 aging, 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 instrumental system (chamber) is part of the measurement instrument, separated from the environment as an isolated, closed, open, isothermal or non-isothermal system (**Table 4**). Reporting O_2 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 instrumental chamber (**Figure 5**). Metabolic O_2 flow per object, $I_{O_2/\underline{N}X}$, is the total O_2 flow in the system divided by the number of objects, N_X, in the system. $I_{O_2/\underline{N}X}$ increases as the mass of the object is increased. Sample mass-specific O_2 flux, $J_{O_2/\underline{m}X}$ should be independent of the mass-concentration of the subsample obtained from the same tissue or cell culture, but system volume-specific O_2 flux, J_{V,O_2} (per liquid volume of the instrumental chamber), increases in proportion to the mass of the sample in the chamber. Although 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- concentration. There are practical limitations to increasing the mass-concentration of the sample in the chamber, when one is concerned about crowding effects and instrumental time resolution. The wall of the chamber and the enclosed solid stirrer are not counted as part of the chamber volume.

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, $J_{V,rB} = d_r\xi_B/dt \cdot V^{-1}$ [(mol·s^{-1})·L^{-1}]. The *rate of concentration change* is dc_B/dt [(mol·L^{-1})·s^{-1}], where concentration is $c_B = n_B \cdot V^{-1}$. There is a difference between (*1*) J_{V,rO_2} [mol·s^{-1}·L^{-1}] and (*2*) rate of concentration change [mol·L^{-1}·s^{-1}]. These merge into 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_{kO_2} [pmol·s^{-1}]) causes a decline in the amount of O_2 in the system, n_{O_2} [nmol]. Normalization of these quantities for the volume of the system, V [L ≡ dm^3], yields volume-specific O_2 flux, $J_{V,kO_2} = I_{kO_2}/V$ [nmol·s^{-1}·L^{-1}], and O_2 concentration, [O_2] or $c_{O_2} = n_{O_2} \cdot V^{-1}$ [µmol·L^{-1} = µM = nmol·mL^{-1}]. Instrumental background O_2 flux is due to external flux into a non-ideal closed respirometer, so 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_{V,kO_2} is relevant mainly for methodological reasons and should be compared with the accuracy of instrumental resolution of background-corrected flux, *e.g.*, ±1 nmol·s^{-1}·L^{-1} (Gnaiger 2001). 'Catabolic' indicates O_2 flux, J_{kO_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*) *Rox* 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 5**), 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 that propose the use of SI units.

Although volume is expressed as m^3 using the SI base unit, the litre [dm^3] is a conventional unit of volume for concentration and is used for most solution chemical kinetics. If one multiplies $I_{O_2/\underline{N}ce}$ by $C_{\underline{N}ce}$, then the result will not only be the amount of O_2 [mol] consumed per time [s^{-1}] in one litre [L^{-1}], but also the change in O_2 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 $mol \cdot L^{-1}$ (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 non-nucleated platelets.

For studies of cells, we recommend that respiration be expressed, as far as possible, as: (*1*) O_2 flux normalized for a mitochondrial marker, for separation of the effects of mitochondrial quality and content on cell respiration (this includes *FCR*s as a normalization for a functional mitochondrial marker); (*2*) O_2 flux in units of cell volume or mass, for comparison of respiration of cells with different cell size (Renner *et al* 2003) and with studies on tissue preparations, and (*3*) O_2 flow in units of attomole (10^{-18} mol) of O_2 consumed per second by each cell [$amol \cdot s^{-1} \cdot cell^{-1}$], numerically equivalent to [$pmol \cdot s^{-1} \cdot 10^{-6}$ cells]. This convention allows information to be easily used when designing experiments in which O_2 flow must be considered. For example, to estimate the volume-specific O_2 flux in an instrumental chamber that would be expected at a particular cell number concentration, one simply needs to multiply the flow per cell by the number of cells per volume of interest. This provides the amount of O_2 [mol] consumed per time [s^{-1}] per unit volume [L^{-1}]. At an O_2 flow of 100 $amol \cdot s^{-1} \cdot cell^{-1}$ and a cell concentration of 10^9 $cells \cdot L^{-1}$ (10^6 $cells \cdot mL^{-1}$), the volume-specific O_2 flux is 100 $nmol \cdot s^{-1} \cdot L^{-1}$ (100 $pmol \cdot s^{-1} \cdot mL^{-1}$).

ET-capacity in human cell types including HEK 293, primary HUVEC, and fibroblasts ranges from 50 to 180 $amol \cdot s^{-1} \cdot cell^{-1}$, measured in living cells in the noncoupled state (Gnaiger 2020). At 100 $amol \cdot s^{-1} \cdot cell^{-1}$ corrected for *Rox*, the current across the mt- membranes, I_{H^+e}, approximates 193 $pA \cdot cell^{-1}$ or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of -110 W. Modelling approaches illustrate the link between protonmotive force and currents (Willis *et al* 2016).

We consider isolated mitochondria as powerhouses and proton pumps as molecular machines to relate experimental results to energy metabolism of living cells. The cellular P»/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 O_2 consumed in the complete oxidation of a mol glycosyl unit (Glyc). Adding 0.5 to the mitochondrial P»/O_2 ratio of 5.4 yields a bioenergetic cell physiological P»/O_2 ratio close to 6. Two NADH equivalents are formed during glycolysis and transported from the cytosol into the mitochondrial matrix, either by the malate-aspartate shuttle or by the glycerophosphate shuttle (**Figure 1a**) 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»/O_2 ratio not only reflects proton translocation and OXPHOS studied in isolation, but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger 1993a).

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 counterparts of cellular core energy metabolism. An O_2 flux balance scheme illustrates the relationships and general definitions (**Figures 1 and 2**).

Box 2: Recommendations for studies with mitochondrial preparations

- Normalization of respiratory rates should be provided as far as possible:

A. Sample normalization

1. *Object-specific biophysical normalization*: on a per organism or per cell basis as O_2 flow; this may not be possible when dealing with coenocytic organisms, *e.g.*, filamentous fungi, or tissues without cross-walls separating individual cells, *e.g.*, muscle fibers.
2. *Size-specific cellular normalization*: per g protein; per organism-, cell- or tissue-mass as mass-specific O_2 flux; per cell volume as cell volume-specific flux.
3. *Mitochondrial normalization*: per mitochondrial marker as mt-specific flux.

B. Chamber normalization

1. Chamber volume-specific flux, J_V [pmol·s^{-1}·mL^{-1}], is reported for quality control in relation to instrumental sensitivity and limit of detection of volume-specific flux.
2. Sample concentration in the instrumental chamber is reported as number concentration, mass concentration, or mitochondrial concentration; this is a component of the measuring conditions. 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 2020). 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 O_2 consumption. Fluxes in mitochondrial coupling states should be, as far as possible, corrected for residual O_2 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 exlcude a shift in substrate competition that may occur when providing physiological substrate cocktails.
- In studies of isolated mitochondria, the mitochondrial recovery and yield should be reported. Experimental criteria such as transmission electron microscopy 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 development of a consistent theory of bioenergetics and mitochondrial physiology. Technical terms related to and defined with normal words can be used as index terms in data repositories, support the creation of ontologies towards semantic information processing (MitoPedia), and help in communicating analytical findings as impactful data-driven stories. '*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 further 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 physiological 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.

Experimentally, respiration is separated in mitochondrial preparations from the interactions with the fermentative pathways of the living cell. OXPHOS analysis is based on the study of mitochondrial preparations complementary to bioenergetic investigations of (*1*) submitochondrial particles and molecular structures, (*2*) living cells, and (*3*) 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_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 5**).

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 are focused on studies using mitochondrial preparations (**Box 2**). These will be extended in a series of reports on pathway control of mitochondrial respiration, respiratory states and rates in living cells, respiratory flux control ratios, and harmonization of experimental procedures.

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