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Mitochondrial respiratory control:

a conceptual perspective on coupling states in mitochondrial preparations.

MITOEAGLE recommendations Part 1

MITOEAGLE Terminology Group

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Mitochondrial respiratory control: MITOEAGLE recommendations 1

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Abstract

Clarity of concepts and consistency of nomenclature is a hallmark of the quality of a research area across its specializations, aimed at facilitating transdisciplinary communication and teaching. The expanding field of mitochondrial respiratory physiology can benefit from harmonization of nomenclature on mitochondrial respiratory states and rates. Peter Mitchell's protonmotive (Δp_m), the chemiosmotic force across the inner mitochondrial membrane, ~~Δp_m~~, establishes the link between electron transfer and phosphorylation of ADP to ATP, and between the electrical and chemical components of energy transformation (ΔpH and $\Delta\Psi_m$). This unifying concept provides the framework for developing a consistent terminology on mitochondrial physiology and bioenergetics. We follow IUPAC guidelines on general terms of physical chemistry, extended by concepts of nonequilibrium thermodynamics and open systems. The differential nomenclature of respiratory sates in classical bioenergetics (States 1 to 5 in an experimental protocol) is incorporated into a concept-driven constructive terminology to address the basic meaning of each respiratory state and focus primarily on the conceptual 'why' with clarification of the experimental 'how'. LEAK states are evaluated to study arrested respiration, *L*, when oxygen consumption compensates mainly for the proton leak. OXPHOS capacity, *P*, is measured at saturating concentrations of ADP and inorganic phosphate to obtain kinetic reference values for diagnostic applications. The ETS state differentiates the oxidative capacity of the electron transfer system, *E*, from *P*, revealing the limitation of OXPHOS capacity by the capacity of the phosphorylation system. Indeed, the d~~D~~evelopment of databases on mitochondrial respiratory control requires the application of strictly defined terms for comparison of respiratory states.

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

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1. Introduction

Every study of mitochondrial function and disease in tissues and cells is faced with **e**volution, **a**ge, **g**ender, **l**ifestyle and **e**nvironment (EAGLE) as essential background conditions characterizing the individual patient or subject, cohort, species, tissue and even cell line. Only a large and coordinated network ~~can manage~~is able to generate the necessary type, quality and number of consistent data sets to address this intrinsic complexity. The global MITOEAGLE network aims at developing harmonized experimental protocols and implementing a quality control and data management system to interrelate results obtained in different studies and generate a rigorously monitored database on mitochondrial respiratory function.

Reliability and comparability of quantitative results depends on accuracy of measurement under well-defined conditions. A conceptually meaningful framework is required to relate the results carried out by different research groups. Vague or ambiguous terminology can lead to confusion and may turn valuable signals into wasteful noise. For this reason, measurements must be expressed in common units on each well-defined attribute for mitochondrial respiratory control. Even if standardization of nomenclature remains a goal of the optimist and is out of reach in the real world, harmonization of the technical jargon will improve the awareness of the intricate meaning of divergent scientific vocabulary. The MITOEAGLE Terminology Group aims at accomplishing the ambitious goal to harmonize, unify and thus simplify the terminology in the field of mitochondrial physiology. A focus on coupling states in mitochondrial preparations may be considered as a first step in the attempt to elaborate a harmonized and conceptually oriented nomenclature in bioenergetics and mitochondrial physiology.

Mitochondrial preparations, mtprep, are defined as tissue or cell preparations in which the plasma membranes are either removed (isolated mitochondria, imt), or mechanically and chemically permeabilized (tissue homogenate, thom; permeabilized fibres,

pfi; or permeabilized cells, pce), while the mitochondrial functional integrity and to a large extent the mitochondrial ~~mt~~ structure ~~are~~ is maintained.

Mitochondria, mt (Greek mitos: thread; chondros: granule) are small structures with a double membrane within cells, which function in cell respiration as powerhouses or ~~batteries~~generators. Mitochondria belong to the bioblasts of Richard Altmann (1894), which comprise mitochondria and symbiotic as well as free-living bacteria. Abbreviation: mt, as generally used in mtDNA. Singular: mitochondrion; plural: mitochondria.

2. Fundamental respiratory coupling states in mitochondrial preparations

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

Mitochondrial respiratory control is exerted in mt ~~preparations~~prep by experimental conditions defined as respiratory states of oxidative phosphorylation. In coupling states the phosphorylation of ADP to ATP is stimulated or depressed, which causes an increase or decrease of the electron flow linked to oxygen consumption. Alternatively, coupling of electron flow and phosphorylation is disengaged by ~~uncouplers which~~uncouplers, which induces a burst of oxygen consumption without performance of biochemical work (Fig. 1). Coupling states in mt-prep~~arations~~ depend on exogenous supply of fuel substrates and oxygen to support the electron transfer system (Fig. 2).

Phosphorylation, »P: Although 'phosphorylation' in the context of OXPHOS is clearly defined as phosphorylation of ADP to ATP, substrate level phosphorylation may be involved as part of the tricarboxylic acid cycle (succinate-CoA ligase) and in the matrix (phosphoenolpyruvate carboxykinase). On the other hand, the term phosphorylation is used in the general literature in many different contexts (phosphorylation of enzymes, etc.). This

justifies consideration of a symbol more discriminative than P as used in the P/O ratio (phosphate to oxygen~~O~~ ratio), where P indicates phosphorylation of ADP to ATP or GDP to GTP. We might consider the symbol »P for the energetic uphill direction of phosphorylation coupled to catabolic reactions, and likewise the symbol «P for the corresponding downhill reaction (Fig. 1).

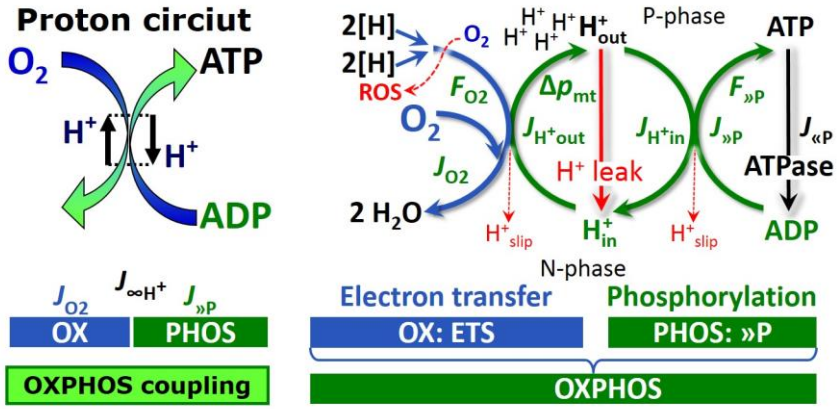

Figure 1. The proton circuit and coupling in oxidative phosphorylation (OXPHOS). Oxygen flux, J_{O2}, is coupled to the phosphorylation of ADP to ATP, $J_{»P}$, by the proton pumps of the electron transfer system (ETS) pushing the outwards proton flux, J_{H+out}. The ATP synthase is driven by the protonmotive force, Δp_{mt}, and inwards proton flux, J_{H+in}, to phosphorylate ADP to ATP. 2[H] indicates the reduced hydrogen equivalents of fuel substrates providing the chemical input force or molar Gibbs energy, F_{O2} [kJ/mol O_2], of the reaction with oxygen, typically in the range of -460 to -480 kJ/mol. The output force is given by the phosphorylation potential, $F_{»P}$ [kJ/mol ADP phosphorylated to ATP], which varies *in vivo* in the range of about 48 to 62 kJ/mol under physiological conditions. Proton turnover, $J_{\infty H+}$, and ATP turnover, $J_{\infty P}$, proceed in the steady state at constant Δp_{mt}, when $J_{\infty H+} = J_{H+out} = J_{H+in}$, and at constant phosphorylation potential, $F_{»P}$, when $J_{\infty P} = J_{»P} = J_{«P}$. $J_{»P}/J_{O2}$ is two times the 'P/O' (»P/O) ratio of classical bioenergetics. The effective $J_{»P}/J_{O2}$ ratio is diminished by (i) the proton leak across the inner mt-membrane from low pH in the positive P-phase to the

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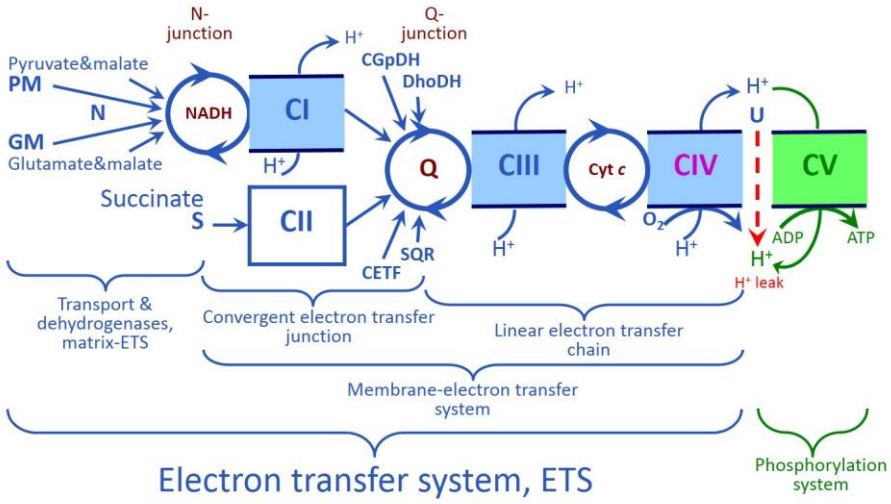
negative N-phase, (ii) cycling of other cations, (iii) proton slip of the proton pumps, and (iv) electron leak generating reactive oxygen species, ROS. Modified after Gnaiger (2014).



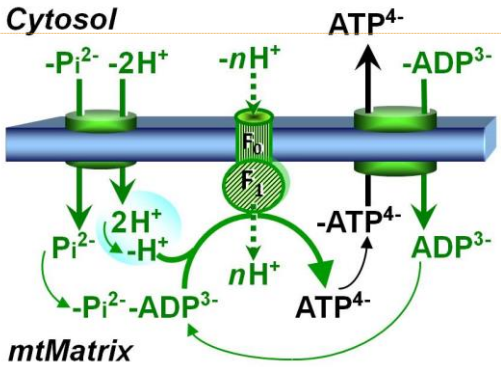


Figure 2: The mitochondrial respiratory system. In oxidative phosphorylation the electron transfer system (A) is coupled to the phosphorylation system (B). Modified after (A) Lemieux et al (2017) and (B) Gnaiger (2014).

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2.1. *Classical terminology for isolated mitochondria ~~(imt)~~*

Kommentiert [JI1]: Already defined

> *'It is essential to define both the substrate and ADP levels in order to identify the steady-state condition of the mitochondria during the experiment'* (Chance and Williams, 1956).

Five classical states of mitochondrial respiration and cytochrome redox states have been introduced by Chance and Williams (1955; 1956). Table 1 explains a protocol with isolated

mitochondria ~~(imt)~~ in a closed respirometric chamber, defining a consecutive sequence of respiratory states.

Kommentiert [JI2]: Already defined before

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

State 2 is induced by addition of a high concentration of ADP, which stimulates respiration transiently on the basis of endogenous fuel substrates, followed by a low respiratory activity limited by endogenous fuel substrate availability.

State 3 is the state stimulated by addition of fuel substrates while the ADP content is still high and supports coupled energy transformation in oxidative phosphorylation.

State 4 is only reached if the imt preparation is of high quality and is well coupled. Depletion of ADP by phosphorylation to ATP will then lead to a decline in oxygen uptake in the transition from State 3 to State 4. Under these conditions a maximum Δp_{mt} and high ATP/ADP ratio are maintained. State 4 respiration reflects intrinsic proton leak and ATPase activity.

State 5 is a minimum oxygen concentration state in a closed respirometric system where oxygen backdifussion may be a cofounding factor preventing complete anoxia.

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

State	$[O_2]$	[ADP]	[Substrate]	Respiration rate	Rate-limiting substance
1	>0	Low	Low	Slow	ADP
2	>0	High	~0	Slow	Substrate
3	>0	High	High	Fast	respiratory chain
4	>0	Low	High	Slow	ADP
5	<0	High	High	0	Oxygen

2.2. Three fundamental coupling states of mitochondrial preparations and residual oxygen consumption

It has been suggested to extend the differential nomenclature (States 1 to 5) by a concept-driven terminology carrying explicit information on the nature of the respiratory states. This terminology must be general and not restricted to any particular experimental protocol or mt-prep~~aration~~. This provides a platform to expand the classical respiratory states with the extended constructive framework in the theory of mitochondrial physiology.

LEAK state (Fig. 3): A state of mitochondrial respiration when oxygen flux is maintained mainly to compensate for the proton leak in the absence of ATP synthesis, in the presence of fuel substrates and oxygen. The LEAK state can be established either in the absence of adenylates, after depletion of ADP at maximum ATP/ADP ratio, ~~and~~ or after inhibition the phosphorylation system by an inhibitor such as oligomycin or carboxyatractyloside. State 4 represents an overestimation of LEAK respiration if ATPase activity prevents final accumulation of ATP and maintains a continuous stimulation of respiration by recycled ADP at $J_{»P}>0$. This can be tested by inhibition of the phosphorylation system using oligomycin, ensuring that $J_{»P}=0$.

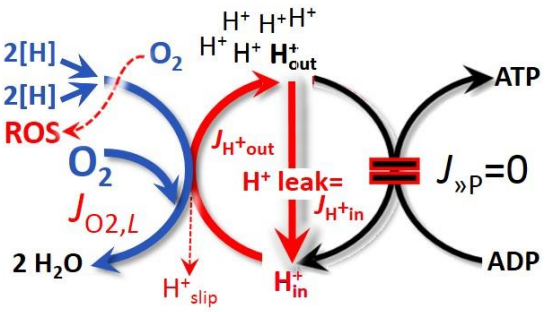

Fig. 3: LEAK state when phoshorylation is arrested, $J_{»P}=0$, and LEAK oxygen flux, $J_{O2,L}$, is controlled mainly by the proton leak, which equals $J_{H^+in,}$ at maximum Δp_{mt} (compare Fig. 1).

OXPHOS state (Fig. 4): In mitochondrial physiology and pathology, maximal mitochondrial respiration in the coupled state is measured for quantitative determination of oxidative phosphorylation (OXPHOS) capacity (Gnaiger 2009). The OXPHOS state is supported by kinetically saturating ADP, [ADP]*, and inorganic phosphate concentrations, [Pi]*, in the presence of fuel substrates and oxygen. The definition of State 3 lacks a fundamental attribute of OXPHOS capacity. As previously highlighted, 'high ADP' in State 3 is a concentration of ADP specifically selected to allow the measurement of a State 3 to State 4 transitions of isolated mitochondria in a closed respirometric system. Starting at oxygen concentrations near air saturation, the ADP concentration added must be low enough to allow phosphorylation to ATP at a coupled oxygen consumption that does not lead to oxygen depletion during the transition to State 4. In contrast, OXPHOS capacity requires evaluation of kinetically saturating ADP concentrations, which are usually an order of magnitude higher than 'high ADP'.

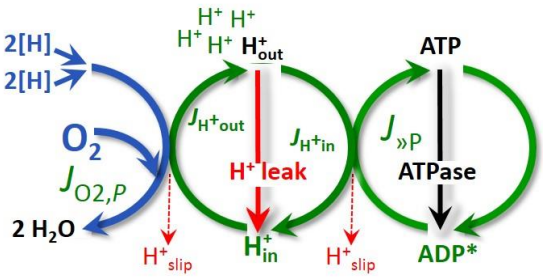

Fig. 4: OXPHOS state when phoshorylation, $J_{»P}$, is supported by a high Δp_{mt}, is stimulated by saturating [ADP]* and [Pi]*, and oxygen flux, $J_{O2,P}$, is highly coupled at a maximum »P/O_2 ratio, $J_{»P}/J_{O2,P}$ (compare Fig. 1).

As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated mitochondria (Puchowicz et al., 2004; Gnaiger, 2001), and even higher ADP concentrations are required particularly in permeabilized fibres to overcome limitations by diffusion and by the tubulin-regulated conductance of the outer mitochondrial membrane (Rostovtseva et al., 2008). In permeabilized muscle fibre bundles of high

respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks et al., 1998). This implies that >90 % saturation is reached only at >5 mM ADP.

ETS state (Fig. 5): The electron transfer system state is the noncoupled state at optimal uncoupler concentration for maximum oxygen flux as a measure of ETS capacity, in the presence of fuel substrates and oxygen. $J_{»P}$=0 for a different reason than in the LEAK state. The abbreviation State 3u is used frequently in bioenergetics, to indicate the state of maximum respiration without sufficient emphasis on the fundamental difference between OXPHOS capacity (coupled, with an uncoupled component) and ETS capacity (noncoupled).

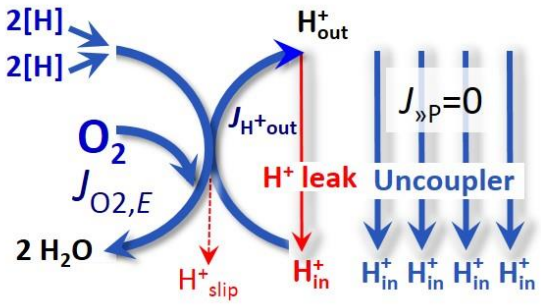

Fig. 5: ETS state when noncoupled respiration, $J_{O2,E}$, is maximum at optimum uncoupler concentration and phosphorylation is zero, $J_{»P}$=0, due to the nearly collapsed Δp_{mt} (compare Fig. 1).

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

3. States and rates

3.1. The steady-state

Steady-state variables (i.e. membrane potential; redox states) and metabolic fluxes (i.e. rates) are measured in defined mitochondrial respiratory states. Steady states can be obtained in open systems, in which changes due to internal transformations (e.g. oxygen consumption) are instantaneously compensated by external flows (e.g. oxygen supply), such that oxygen concentration does not change in the system (Gnaiger 1993). Mitochondrial respiratory states monitored in closed systems may satisfy the criteria of pseudo-steady states for limited periods of time, when the changes occurring in the system (oxygen concentration, fuel substrate concentration, [ADP]) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Pseudo-steady states require saturating levels of substrates to be maintained and thus depend on the kinetics of the processes under investigation.

Protonmotive force, Δp_{mt}: The protonmotive force is maximum in the LEAK state of ~~well-coupled~~well-coupled mitochondria, driven by LEAK respiration and maintained high at a minimum backflux of protons to the matrix side. Δp_{mt} is high in the OXPHOS state when it drives phosphorylation, and very low in the ETS state when uncouplers ~~shortcircuit~~short-circuit the proton cycle. The protonmotive force, Δp_{mt},

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

is composed of an electric part, $\Delta \Psi_{mt}$, which is the potential difference across the inner mt-membrane, and a chemical part, $\Delta \mu_{H+}/F$, which stems from the difference of pH across the mt-membrane. Protonmotive means that the proton is moved across the mt-membrane at ΔpH maintained across the mt-membrane,

$$\Delta \mu_{H+} = -2.3 \cdot RT \cdot \Delta pH \quad (2)$$

where RT is the gas constant times absolute temperature (RT = 2.279 and 2.579 kJ·mol^{-1} at 25 and 37 °C, respectively). The Faraday constant, $F = N_A$, is the product of the elementary charge, e [C] and the Avogadro (or Loschmidt) constant. F yields the conversion between

electric force expressed in joules per coulomb or Volt [V=J/C] and chemical force, $\Delta\mu_{H+}$, with the unit joules per mole [J/mol].

Forces and flows in physics and irreversible thermodynamics: According to the definition in physics, if we define the protonmotive force, Δp_{mt}, ~~is not a force. A~~ as a potential difference is not a force of physics (Cohen et al 2008). Complementary to the attempt of a unification of fundamental forces defined in physics, the style of thinking of Nobel laureates Lars Onsager, Erwin Schrödinger, Ilya Prigogine and Peter Mitchell (even if expressed in apparently unrelated forms) is related to the diversity of conjugated flow-force relationships, the product of which yields the dissipation function linked to the Second Law of thermodynamics (Prigogine 1967; Schrödinger 1944). The concept of driving forces is embedded in statistical and irreversible thermodynamics, and the fundamental forces of physics are distinguished from the generalized forces of thermodynamics. A generalized force is the change of potentially available or 'free' energy (exergy) per 'motive' unit. A potential difference is, in the framework of flow-force relationships, a generalized force, F_{tr}, involved in an exergy transformation, defined as the partial derivative of Gibbs energy per advancement, $d_{tr}\xi$, of the transformation, tr: $F_{tr} = \partial_{tr}G/\partial_{tr}\xi$ (Gnaiger 1993). In chemical reactions and osmotic or diffusion processes occurring in a closed heterogenous system, such as a chamber containing isolated mitochondria, scalar transformations occur without defined spacial direction but between defined compartments (translocation between the matrix and extramitochondrial space) or between energetically defined chemical substances (reactions from substrates to products). The corresponding fluxes are not vectorial but scalar, and are expressed per volume and not per cross-sectional area. The corresponding generalized forces are scalar, expressed in the unit [J·mol^{-1}]. In a scalar electric transformation (flux of charge or current from the matrix space to the intermembrane and extramitochondrial space) the effective force is the mt-membrane potential, $\Delta\Psi_{mt}$ [V=J·C^{-1}]. For comparison, in a mechanical, vectorial advancement, $d_{me}\xi$ [m], the unit of the force is newton, $\boldsymbol{F}_{me}$ [N=J·m^{-1}],

and the 'flow' is the velocity, $\boldsymbol{v}$ = $d_{me}\xi/dt$ [$m \cdot s^{-1}$], such that the flow·force product yields mechanical power, P_{me} [W] (Cohen et al 2008). The corresponding vectorial 'flux' (flow density per area) is velocity per cross-sectional area [$s^{-1} \cdot m^{-1}$]. The scalar 'flux' lacks spacial information in a given volume, such that flux (volume-density [$s^{-1} \cdot m^{-2}$]) times force yields power density (per volume), P_{Vme} [$W \cdot m^{-3}$].

Coupling, efficiency and power: In energetics (ergodynamics) coupling is defined as an exergy transformation fuelled by the advancement of an exergonic (downhill) input process driving the advancement of an endergonic (uphill) output process. The (negative) output/input power ratio is the efficiency of a coupled energy transformation. Power, P_{tr} = $\partial_{tr}G/dt$ [$W=J \cdot s^{-1}$], is closely linked to the dissipation function (Prigogine 1967) and is the product of flow, $I_{tr}=d_{tr}\xi \cdot dt^{-1}$ [$x_{tr} \cdot s^{-1}$] times generalized force, $F_{tr} = \partial_{tr}G/\partial_{tr}\xi$ [$J \cdot x_{tr}^{-1}$] (Gnaiger 1993).

3.2. Normalization: flows and fluxes

Application of common units is required for direct transfer of reported results into a database. The second [s] is the *SI* unit for the base quantity time. The term 'rate' is too general and not useful for a database (Fig. 6).

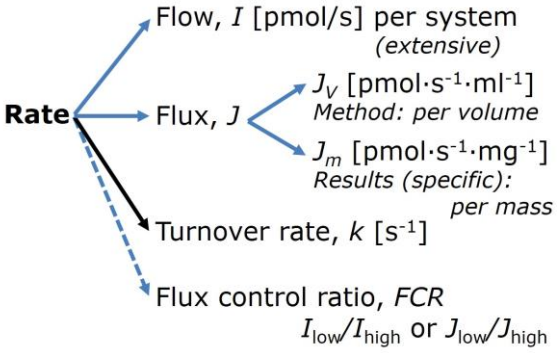

Figure 6: Different meanings of rate may lead to confusion, if the normalization is not sufficiently specified. Results are frequently expressed as mass-specific flux, J_m, per mg protein, dry or wet weight, ignoring the fundamental difference between weight and mass. Cell volume or mitochondrial volume may be used for normalization (volume-specific flux, J_{Vce} or J_{Vmt}),

which then must be clearly distinguished from flux, J_V, expressed for methodological reasons per volume of the measurement chamber.

Flow, *I*, per instrumental system: In analogy to electric terms, flow as an extensive quantity (*I*; per system) is distinguished from flux as a size-specific quantity (*J*; per system size) (Fig. 6). Electric current is a flow, I_{el} [$A = C \cdot s^{-1}$], per system (extensive quantity).

Flux per volume of the experimental system, J_V: When dividing an extensive quantity by system size (the cross-sectional area of a wire or area of a membrane), a size-specific quantity is obtained, which is electric flux (electric current density), $\boldsymbol{J}_{el}$ [$A \cdot m^{-2} = C \cdot s^{-1} \cdot m^{-2}$]. In open systems, external flows (such as oxygen supply) are distinguished from internal transformations (metabolic flow, oxygen consumption). In a closed system external flows of all substances are zero, and the internal flow of oxygen consumption, I_{O2} [$pmol \cdot s^{-1}$], causes a decline of the amount of oxygen in the system, n_{O2} [nmol]. Normalization of these quantities for the volume of the system, V [ml = cm^3] yields oxygen flux, $J_{V,O2} = I_{O2}/V$ [$pmol \cdot s^{-1} \cdot ml^{-1}$] and oxygen concentration, [O_2] or $c_{O2} = n_{O2}/V$ [$nmol \cdot ml^{-1} = \mu mol \cdot l^{-1} = \mu M$]. Volume-specific metabolic oxygen flux, $J_{V,O2}$, depends on the specific activity and the concentration of the mt-preparation in the measurement system, mtprep/*V*.

Size-specific flux, *J*: $J_{V,O2}$ should be compared with instrumental resolution and is thus relevant mainly for methodological reasons. Normalization for mtprep/*V* is required for reporting respiratory results, e.g. in terms of respiration per mass, W_{mtprep} (of tissue homogenate or permeabilized fibres), $J_{O2} = J_{V,O2}/(W_{mtprep}/V) = I_{O2}/W_{mtprep}$.

Flow, *I*, per experimental model: A special case of normalization is encountered in respiratory studies with permeabilized (or intact) cells. If respiration is expressed per million cells, the extensive quantity of oxygen flow per measurement system is replaced by the extensive quantity of oxygen flow, I_{O2}, per cell (or per 10^6 cells). Similarly, oxygen flow can be calculated from volume-specific oxygen flux, $J_{V,O2}$ [$pmol \cdot s^{-1} \cdot ml^{-1}$] (per V of the

measurement chamber), divided by the number density of cells, $C=N_{ce}/V$ [10^6·ml^{-1}], where N_{ce} is the number of cells in the chamber. Cellular oxygen flow can be compared only between cells of identical cell size, after normalization to a size-specific oxygen flux or after normalization by a mt-marker (Renner et al, 2003).

3.3. Conversion: oxygen, protons, ATP

J_{O2} is coupled in mitochondrial steady states to proton cycling, $J_{\infty H+} = J_{H+out} = J_{H+in}$ (Fig. 1). J_{H+out} [pmol·s^{-1}·ml^{-1}] is converted into an electric flux (per volume), J_{el} [µC·s^{-1}·ml^{-1}=µA·ml^{-1}] = J_{H+out} [pmol·s^{-1}·ml^{-1}]·F [C·mol^{-1}]·10^{-6}. F is the Faraday constant (96,485.3 C·mol^{-1}). At a J_{H+out}/J_{O2} ratio or H^+_{out}/O_2 of 20 (H^+_{out}/O=10), a volume-specific oxygen flux of 100 pmol·s^{-1}·ml^{-1} would correspond to a proton flux of 2,000 pmol H^+_{out}·s^{-1}·ml^{-1} or volume-specific current of 193 µA·ml^{-1}.

$$J_{el}\ [\mu A\cdot ml^{-1}] = J_{H+out}\cdot F\cdot 10^{-6}\ [pmol\cdot s^{-1}\cdot ml^{-1}\cdot \mu C\cdot pmol^{-1}] \quad (3.1)$$

$$J_{el}\ [\mu A\cdot ml^{-1}] = J_{V,O2}\cdot (H^+_{out}/O_2)\cdot F\cdot 10^{-6}\ [\mu A\cdot ml^{-1}=\mu C\cdot s^{-1}\cdot ml^{-1}] \quad (3.2)$$

ETS capacity in various human cell types including HEK 293, primary HUVEC and fibroblasts ranges from 50 to 180 pmol·s^{-1}·10^{-6} cells (see Gnaiger 2014). At 100 pmol·s^{-1}·10^{-6} cells corrected for ROX, the current across the mt-membranes, I_{el}, approximates 193 µA·10^{-6} cells or 0.2 nA per cell.

In the OXPHOS state or at high [ADP], J_{H+in} drives a phosphorylation flux, $J_{V,»P}$, of 500 pmol·s^{-1}·ml^{-1} at a H^+_{in}/»P ratio of 3.7 (*n*H+; Fig. 2B).

For NADH- and succinate-linked respiration, the mechanistic »P/O_2 ratio is calculated at 20/3.7 and 12/3.7 (Eq 2), or 5.4 and 3.3 (equivalent to »P/O of 2.7 and 1.6; Watt et al 2010), in direct agreement with the measured »P/O ratio for succinate of 1.58 ± 0.02 (Gnaiger et al 2000),

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

In summary,

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

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

4. Conclusions

With a perspective to extend the present recommendations on coupling control (part 1) to pathway control of mitochondrial respiration (part 2), substrate-uncoupler-inhibitor-titration (SUIT) protocols, and harmonization of experimental procedures, MITOEAGLE will be a gateway and milestone to better diagnose mitochondrial respiratory defects which are linked to genetic variations, age-related health risks, gender-specific mitochondrial performance, life style with its consequences on degenerative diseases, and environmental exposure to toxicological agents.

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If we want a list of selected terms and symbols it has to be a table.

Table 1. Definition of terms and symbols

Symbol	Additions[1]	Definition
ETS, state *E*	Substrates, uncoupler	Noncoupled state at optimal uncoupler concentration for maximum oxygen flux as a measure of electron transfer system capacity (State 3u)
OXPHOS, state *P*	Substrates, ADP, P_i	ADP activated state at maximum oxygen flux as a measure of the capacity for oxidative phosphorylation (compare State 3)
LEAK, state *L*	Substrates; no ADP; oligomycin, atractyloside	Resting state of non-phosphorylating respiration when oxygen flux is maintained mainly to compensate for the proton leak after inhibition of ATP synthesis (compare State 4; 4o)
$J_{O2,E}$; $J_{O2,P}$ per tissue		Oxygen flux [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ wet weight] expressing tissue-ETS or tissue-OXPHOS capacity
$J_{O2,E}$; $J_{O2,P}$ per mt-marker		Oxygen flux [nmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ P_{mt}] expressing ETS or OXPHOS capacity per mitochondrial marker
$L/E = J_{O2,L} / J_{O2,E}$		*L/E* coupling control ratio; increases with uncoupling at constant ETS capacity
$P/E = J_{O2,P} / J_{O2,E}$		*P/E* coupling control ratio; decreases with limitation by the phosphorylation system

$L/P = (L/E) / (P/E)$		L/P coupling control ratio; 1/RCR
$FCR = J_i / J_{ref}$		Flux control ratios; fluxes in various states i normalized to a common reference state, J_{ref}
$CCR = J_i / J_{ref}$	Constant substrate	Coupling control ratios, FCR with J_i and J_{ref} in the same pathway control state
ROX	Rotenone+ myxothiazol	Residual oxygen consumption, subtracted from total flux