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The protonmotive force and respiratory control:

Building blocks of mitochondrial physiology

Part 1.

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MitoEAGLE Network

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41 **Updates:**

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

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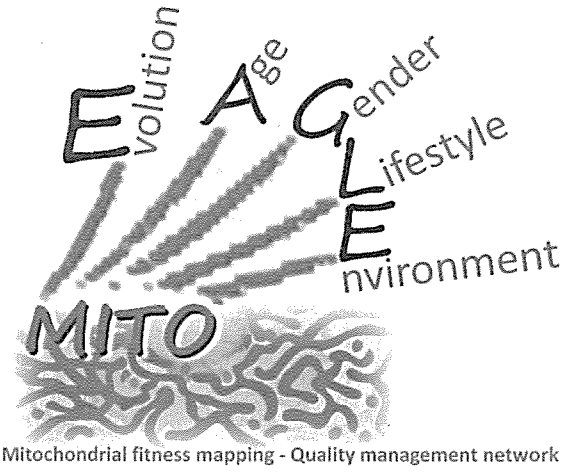
This manuscript on 'The protonmotive force and respiratory control' is a position statement in the frame of COST Action CA15203 MitoEAGLE. The list of co-authors evolved from MitoEAGLE Working Group Meetings and a ***bottom-up*** *spirit of COST in phase 1: This is an open invitation to scientists and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



Phase 2 - until ***October 12:*** *We continue to invite comments and suggestions on the MitoEAGLE preprint, particularly if you are an* ***early career investigator adding an open future-oriented perspective****, or an* ***established scientist providing a balanced historical basis****. Your critical input into the quality of the manuscript will be most welcome, improving our aims to be educational, general, consensus-oriented, and practically helpful for students working in mitochondrial respiratory physiology.*

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

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

*We organize a MitoEAGLE session linked to our series of reports at the MiP*conference *Nov 2017 in Hradec Kralove in close association with the MiPsociety (where you hopefully will attend) and at EBEC 2018 in Budapest.*

» http://www.mitoeagle.org/index.php/MiP2017_Hradec_Kralove_CZ

I thank you in advance for your feedback.
With best wishes,

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Abstract

Clarity of concepts and consistency of nomenclature are trademarks of a research field across its specializations, facilitating transdisciplinary communication and education. As research and knowledge of mitochondrial physiology expand, the necessity for harmonizing nomenclature concerning mitochondrial respiratory states and rates has become apparent. Peter Mitchell's concept of the protonmotive force establishes the links between electrical and chemical components of energy transformation and coupling in oxidative phosphorylation. This unifying concept provides the framework for developing a consistent terminology of mitochondrial physiology and bioenergetics. We follow IUPAC guidelines on general terms of physical chemistry, extended by concepts of open systems and irreversible thermodynamics. We align the nomenclature of classical bioenergetics on respiratory states with a concept-driven constructive terminology to address the meaning of each respiratory state. Standards for evaluation of respiratory states must be followed for the development of databases of mitochondrial respiratory function in species, tissues and cells studied under diverse physiological and experimental conditions.

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

this is a very long paragraph and I suggest dividing into smaller, more specific paragraphs

Box 1:

In brief: mitochondria and Bioblasts

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

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

new paragraph

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

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

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

216

217 **1. Introduction**

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

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

in the attempt to generate a harmonized and conceptually-oriented nomenclature in bioenergetics and mitochondrial physiology. Coupling states of intact cells and respiratory control by fuel substrates and specific inhibitors of respiratory enzymes will be reviewed in subsequent communications.

2. Respiratory coupling states in mitochondrial preparations

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

2.1. Definitions

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

266 detergents leads to the complete loss of cell viability, tested by nuclear staining, while
267 mitochondrial function remains unaffected, as shown by the lack of a respiratory response of
268 respiration of isolated mitochondria to the addition of such low concentrations of digitonin and
269 saponin. Mechanical or chemical permeabilization is applied in tissue homogenates containing
270 all components of the cell in the crude homogenate at highly diluted concentrations. Likewise,
271 in permeabilized tissues or cells the functional and structural integrity of mitochondria are
272 largely maintained. All mitochondria are retained in chemically permeabilized mitochondrial
273 preparations and crude tissue homogenates. In the preparation of isolated mitochondria the cells
274 or tissues are homogenized, and the mitochondria are separated from other cell fractions and
275 purified by centrifugation, entailing the loss of a significant fraction of mitochondria. The term
276 mitochondrial preparation does not include further fractionation of mitochondrial components,
277 as well as submitochondrial particles.

278 **Control and regulation:** The terms metabolic *control* and *regulation* are frequently used
279 synonymously, but are distinguished in metabolic control analysis: 'We could understand the
280 regulation as the mechanism that occurs when a system maintains some variable constant over
281 time, in spite of fluctuations in external conditions (homeostasis of the internal state). On the
282 other hand, metabolic control is the power to change the state of the metabolism in response to
283 an external signal' (Fell 1997). Respiratory control may be induced by experimental control
284 signals that *exert* an influence on: (*1*) ATP demand and ADP phosphorylation rate; (*2*) fuel
285 substrate, pathway competition and oxygen availability, *e.g.*, starvation and hypoxia; (*3*) the
286 protonmotive force, redox states, flux-force relationships, coupling and efficiency; (*4*) Ca^{2+} and
287 other ions including H^+; (*5*) inhibitors, *e.g.*, nitric oxide or intermediary metabolites, such as
288 oxaloacetate. *Mechanisms* of respiratory control and regulation include adjustments of (*1*)
289 enzyme activities by allosteric mechanisms and phosphorylation, (*2*) enzyme content,
290 concentrations of cofactors and conserved moieties (such as adenylates, nicotinamide adenine
291 dinucleotide [NAD^+/NADH], coenzyme Q, cytochrome *c*); (*3*) metabolic channeling by

292 supercomplexes; and *(4)* mitochondrial density (enzyme concentrations and membrane area)
293 and morphology (cristae folding, fission and fusion). (*5*) Mitochondria are targeted directly by
294 hormones, thereby affecting their energy metabolism (Lee *et al.* 2013; Gerö and Szabo 2016;
295 Price and Dai 2016; Moreno *et al.* 2017). Evolutionary or acquired differences in the genetic
296 and epigenetic basis of mitochondrial function (or dysfunction) between subjects and gene
297 therapy; age; gender, biological sex, and hormone concentrations; life style including exercise
298 and nutrition; and environmental issues including thermal, atmospheric, toxicological and
299 pharmacological factors, exert an influence on all control mechanisms listed above (for reviews,
300 see Brown 1992; Gnaiger 1993a, 2009; 2014; Paradies *et al.* 2014; Morrow *et al.* 2017).

301 **Respiratory control and response:** There is a difference between control by a fixed
302 component of a metabolic system or module, e.g. ATP synthase, and the response to an
303 experimental variable, *e.g.*, fuel substrate or ADP. Whilst lack of control by a metabolic
304 module, *e.g.* phosphorylation system, does mean that there will be no response to a variable
305 activating it, *e.g.* [ADP], the reverse is not true; *i.e.*, lack of response to [ADP] does not exclude
306 the phosphorylation system from having some degree of control. The degree of control of a
307 component of the OXPHOS system on an output variable of the system, such as oxygen flux,
308 will in general be different from the degree of control on other outputs, such as phosphorylation
309 flux, cytochrome redox states, protonmotive force, phosphorylation potential, and proton leak
310 flux (Box 2). As such, it is necessary to be specific as to which output is under consideration.
311 Respiratory control is insufficiently specific in the context of specific interpretations (Fell
312 1997).

313 **Respiratory coupling control:** Respiratory control is monitored in a mitochondrial
314 preparation under conditions defined as respiratory states. When phosphorylation of ADP to
315 ATP is stimulated or depressed, an increase or decrease is observed in electron flux linked to
316 oxygen consumption in 'controlled' coupling states in intact mitochondria. Alternatively,
317 coupling of electron transfer with phosphorylation is disengaged by disruption of the integrity

318 of the inner mitochondrial membrane or by uncouplers, functioning like a clutch in a
319 mechanical system. The corresponding coupling control state is characterized by high levels of
320 oxygen consumption without control by phosphorylation ('uncontrolled state'; classical
321 terminology). Energetic coupling is defined in Box 3. Respiratory control refers to the ability
322 of mitochondria to adjust oxygen consumption in response to external control signals by
323 engaging various mechanisms of control and regulation. Loss of coupling by intrinsic
324 uncoupling and decoupling, or pathological dyscoupling, lowers the efficiency. Such
325 generalized uncoupling is different from switching to mitochondrial pathways that involve
326 fewer than three proton pumps ('coupling sites': Complexes CI, CIII and CIV), bypassing CI
327 through multiple electron entries into the Q-junction (**Fig. 1**). A bypass of CIII and CIV is
328 provided by alternative oxidases, which reduce oxygen without proton translocation.
329 Reprogramming of mitochondrial pathways may be considered as a switch of gears (changing
330 the stoichiometry) rather than uncoupling (loosening the stoichiometry).

331 **Pathway control states** are obtained in mitochondrial preparations by depletion of
332 endogenous substrates and addition to the mitochondrial respiration medium of fuel substrates
333 (CHNO) and specific inhibitors, activating selected mitochondrial pathways (**Fig. 1**). Coupling
334 control states and pathway control states are complementary, since mitochondrial preparations
335 depend on an exogenous supply of pathway-specific fuel substrates and oxygen (Gnaiger 2014).
336

337 **Box 2: Metabolic fluxes and flows: vectorial and scalar**

338 In the mitochondrial electron transfer system (**Fig. 1**), vectorial transmembrane proton flux is
339 coupled through the proton pumps CI, CIII and CIV to the catabolic flux of scalar reactions,
340 collectively measured as oxygen flux. In **Fig. 2**, the scalar catabolic reaction, k, of oxygen
341 consumption, $J_{O2,k}$ [mol·s^{-1}·m^{-3}], is expressed as oxygen flux per volume, V [m^3], of the
342 experimental chamber (the system). Fluxes are *vectors*, if they have *spatial* direction in addition
343 to magnitude. A vector flux (surface-density of flow) is expressed per unit cross-sectional area,

344 A [m^2], perpendicular to the direction of flux. If *flows, I,* are defined as extensive quantities of
345 the *system*, as vector or scalar flow, $\boldsymbol{I}$ or I [$mol \cdot s^{-1}$], respectively, then the corresponding vector
346 and scalar *fluxes, J,* are obtained as $\boldsymbol{J}=\boldsymbol{I} \cdot A^{-1}$ [$mol \cdot s^{-1} \cdot m^{-2}$] and $J=I \cdot V^{-1}$ [$mol \cdot s^{-1} \cdot m^{-3}$], respectively,
347 expressing flux as an area-specific vector or volume-specific scalar quantity. Volume-specific
348 scalar O_2 flux is coupled (**Box 3**) to vectorial translocation of protons across the inner
349 mitochondrial membrane, from the negative compartment (matrix space; N-phase) to the
350 positive compartment (inter-membrane space; P-phase; **Fig. 2**). The *scalar or compartmental*
351 direction of a chemical reaction, A → B, is defined by assigning substrates and products, A and
352 B, as energetic 'compartments' (O_2 is defined as a substrate in respiratory O_2 consumption). In
353 direct analogy to A → B, the compartmental direction of a vectorial translocation (*e.g.*
354 diffusion) from the N-phase to the P-phase is defined by assigning the initial and final state as
355 energetic compartments, $H^+_{in} \rightarrow H^+_{out}$, respectively (Gnaiger 1993b). Vectorial transmembrane
356 proton flux, $J_{H+,out}$, is analyzed in a heterogenous compartmental system as a quantity with
357 *directional* but not *spacial* information. In order to establish a quantitative relation between the
358 coupled fluxes, both $J_{O2,k}$ and $J_{H+,out}$ must be expressed in identical units ([$mol \cdot s^{-1} \cdot m^{-3}$] or
359 [$C \cdot s^{-1} \cdot m^{-3}$]), yielding the H^+_{out}/O_2 ratio (**Fig. 1**). The *vectorial* proton flux in compartmental
360 translocation has *compartmental direction*, distinguished from a *vector* flux with *spatial*
361 *direction*. Likewise, the corresponding protonmotive force is defined as an electrochemical
362 potential *difference* between two compartments, which is different from a vector force or
363 *gradient* across the membrane with defined spatial direction.

364

365 *2.2. Three coupling states of mitochondrial preparations and residual oxygen consumption*

366 **Coupling control states:** To extend the classical nomenclature on mitochondrial
367 coupling states (Section 2.3) by a concept-driven terminology that incorporates explicit
368 information on the nature of the respiratory states, the terminology must be general and not
369 restricted to any particular experimental protocol or mitochondrial preparation (Gnaiger 2009).

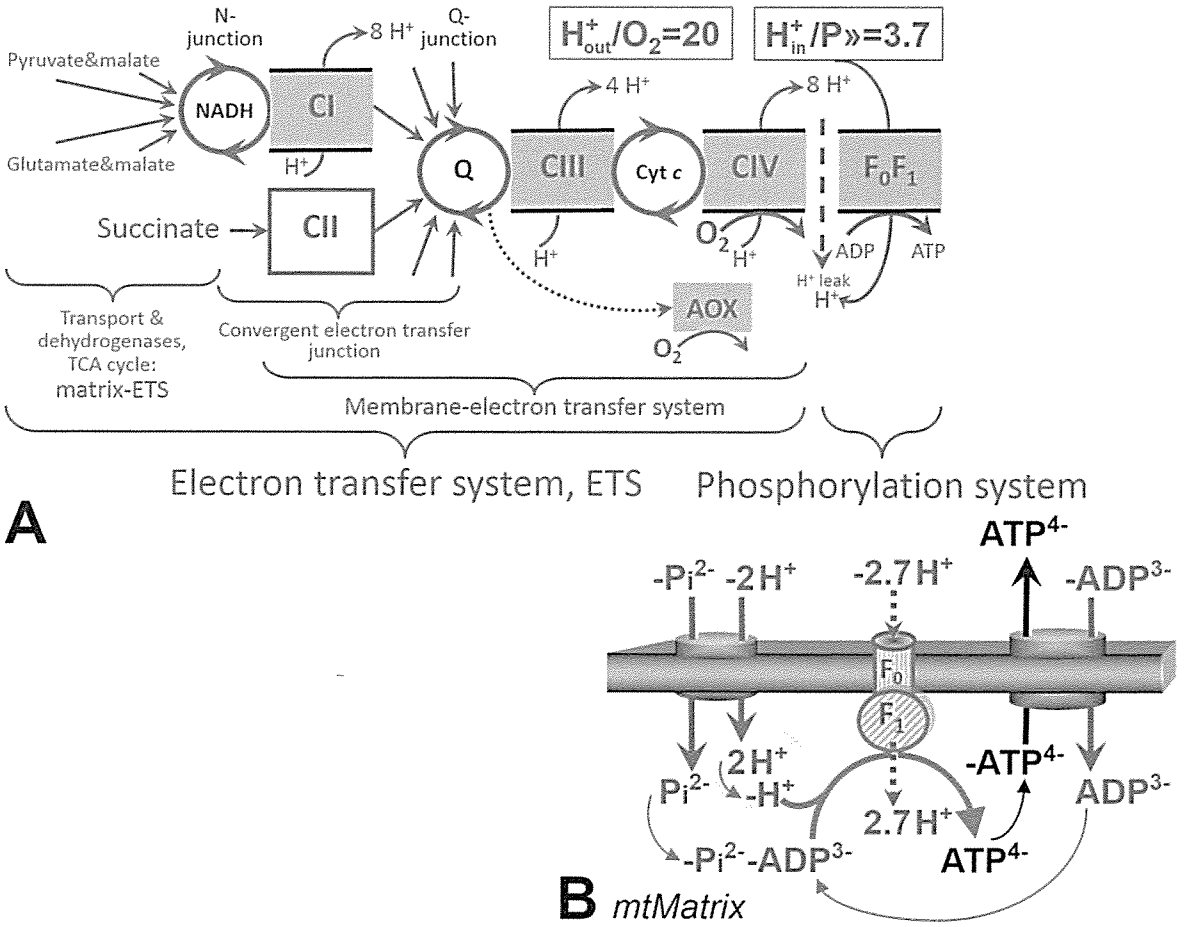
I wonder if 'power' is a better term? For me, 'capacity' is more analagous to 'volume' and doesn't include change per unit of time?

We focus primarily on the conceptual 'why', along with clarification of the experimental 'how'. In the following section, the concept-driven terminology is explained and coupling states are defined. The capacity of *oxidative phosphorylation*, OXPHOS, provides diagnostic reference values for physiological respiratory capacities of defined pathways of core energy metabolism and is, therefore, measured at kinetically-saturating concentrations of ADP and inorganic phosphate, P_i. The *oxidative* capacity of the electron transfer system, ETS, reveals the limitation of OXPHOS capacity mediated by the *phosphorylation* system. ETS capacity is measured as noncoupled respiration by application of *external uncouplers*. The contribution of *intrinsically uncoupled* oxygen consumption is most easily studied by not stimulating or arresting phosphorylation, when oxygen consumption compensates mainly for the proton leak; the corresponding states are collectively classified as LEAK states (**Table 1**). Coupling states of mitochondrial preparations can be compared in any defined mitochondrial pathway control state (**Fig. 1**). Fuel substrates and ETS inhibitors are kept constant while (*1*) adding ADP or P_i, (*2*) inhibiting the phosphorylation system, and (*3*) performing uncoupler titrations.

OK, I've seen below ☺

Respiratory capacities and kinetic control: Coupling control states are established in the study of mitochondrial preparations to obtain reference values for various output variables. Physiological conditions *in vivo* may deviate substantially from these experimentally obtained states. Since kinetically-saturating concentrations, *e.g.* of ADP or oxygen, may not apply to physiological intracellular conditions, relevant information is obtained in studies of kinetic responses to conditions intermediate between the LEAK state at zero [ADP] and the OXPHOS state at saturating [ADP], or of respiratory capacities in the range between kinetically saturating [O_2] and anoxia (Gnaiger 2001). We define respiratory capacities, comparable to channel capacity in information theory, as the upper bound of the rate of respiration measured in defined coupling and pathway control states of mitochondrial preparations (**Box 3**).

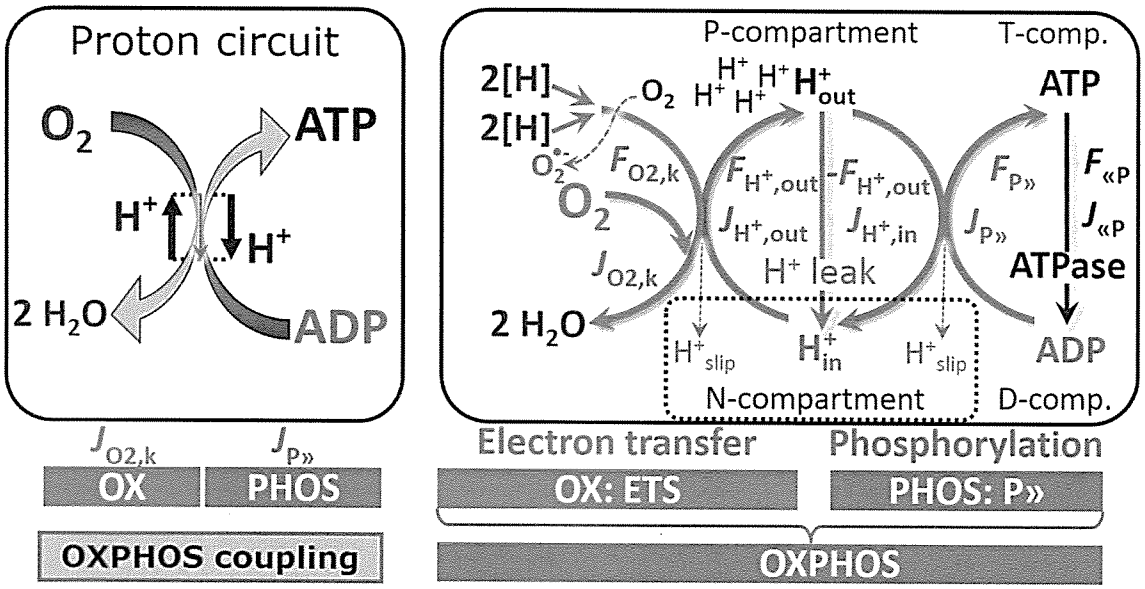
Given my comment above, maybe this definition of capacity needs to be introduced earlier?



395

396 **Fig. 1. The mitochondrial respiratory system and oxidative phosphorylation. (A)** The electron
397 transfer system, ETS, and coupling to the phosphorylation system. Multiple convergent electron transfer
398 pathways are shown from NADH and succinate; additional arrows indicate electron entry through
399 electron transferring flavoprotein, glycerophosphate dehydrogenase, dihydro-orotate dehydrogenase,
400 choline dehydrogenase, and sulfide-ubiquinone oxidoreductase. The branched pathway of oxygen
401 consumption by alternative quinol oxidase (AOX) is indicated by the dotted arrow. H^+_{out}/O_2 is the ratio of
402 outward proton flux from the matrix space to catabolic O_2 flux in the NADH-linked pathway. H^+_{in}/P» is
403 the ratio of inward proton flux from the inter-membrane space to the flux of phosphorylation of ADP to
404 ATP. Due to proton leak and slip these are not fixed stoichiometries. (**B**) Phosphorylation system
405 consisting of the F_1F_0 ATP synthase, adenine nucleotide translocase, and the inorganic phosphate
406 transporter. The H^+_{in}/P» stoichiometry is the sum of the coupling stoichiometry in the ATP synthase
407 reaction (-2.7 H^+ from the intermembrane space, 2.7 H^+ to the matrix) and the proton balance in the
408 translocation of ADP^{2-}, ATP^{3-} and P_i^{2-}. See Eqs. 3 and 4 for further explanation. Modified from (A)
409 Lemieux *et al.* (2017) and (B) Gnaiger (2014).

410



411

412 **Fig. 2. The proton circuit and coupling in oxidative phosphorylation (OXPHOS).** Oxygen flux, $J_{O2,k}$,
413 in a catabolic reaction k is coupled to the phosphorylation of ADP to ATP, $J_{P»}$, by the proton pumps of
414 the electron transfer system, ETS, pushing the outward proton flux, $J_{H+,out}$, and generating the output
415 protonmotive force, $F_{H+,out}$. ATP synthase is coupled to inward proton flux, $J_{H+,in}$, to phosphorylate ADP
416 with inorganic phosphate to ATP, driven by the input protonmotive force, $F_{H+,in}$=-$F_{H+,out}$. 2[H] indicates
417 the reduced hydrogen equivalents of fuel substrates that provide the chemical input force, $F_{O2,k}$ [kJ/mol
418 O_2], of the catabolic reaction k with oxygen (Gibbs energy of reaction per mole O_2 consumed in reaction
419 k), typically in the range of -460 to -480 kJ/mol. The output force is given by the phosphorylation potential
420 difference (ADP phosphorylated to ATP), $F_{P»}$, which varies *in vivo* ranging from about 48 to 62 kJ/mol
421 under physiological conditions. Fluxes, J_B, and forces, F_B, are expressed in either chemical units,
422 [mol·s^{-1}·m^{-3}] and [J·mol^{-1}] respectively, or electrical units, [C·s^{-1}·m^{-3}] and [J·C^{-1}] respectively, per volume,
423 V [m^3], of the system. The system defined by the boundaries shown as a full black line is not a black
424 box, but is analysed as a compartmental system. The negative compartment (N-compartment, enclosed
425 by the dotted line) is the matrix space, separated from the positive compartment (P-compartment) by
426 the inner mitochondrial membrane. ADP+P_i and ATP are the substrate- and product-compartments
427 (scalar D- and T-comp.), respectively. Chemical potentials of all substrates and products involved in the
428 scalar reactions are measured in the P-compartment for calculation of the scalar forces $F_{O2,k}$ and
429 $F_{P»}$=-$F_{«P}$. Modified from Gnaiger (2014).

430

Phosphorylation, P»: *Phosphorylation* in the context of OXPHOS is defined as phosphorylation of ADP to ATP. On the other hand, the term phosphorylation is used generally in many different contexts, *e.g.* protein phosphorylation. This justifies consideration of a symbol more discriminating and specific than P as used in the P/O ratio (phosphate to atomic oxygen ratio), where P indicates phosphorylation of ADP to ATP or GDP to GTP. We propose the symbol P» for the endergonic direction of phosphorylation ADP→ATP, and likewise the symbol «P for the corresponding exergonic hydrolysis ATP→ADP (**Fig. 2**). ATP synthase is the proton pump of the phosphorylation system (**Fig. 1B**). P» may also involve substrate-level phosphorylation as part of the tricarboxylic acid cycle (succinyl-CoA ligase) and phosphorylation of ADP catalyzed by phosphoenolpyruvate carboxykinase, adenylate kinase, creatine kinase, hexokinase and nucleoside diphosphate kinase (NDPK). Kinase cycles are involved in intracellular energy transfer and signal transduction for regulation of energy flux. In isolated mammalian mitochondria ATP production catalyzed by adenylate kinase, 2ADP ↔ ATP + AMP, proceeds without fuel substrates in the presence of ADP (Komlódi and Tretter 2017). $J_{P»}/J_{O2,k}$ (P»/O_2) is two times the 'P/O' ratio of classical bioenergetics. The effective P»/O_2 ratio is diminished by: (*1*) the proton leak across the inner mitochondrial membrane from low pH in the P-phase to high pH in the N-phase (P, positive; N, negative); (*2*) cycling of other cations; (*3*) proton slip in the proton pumps when a proton effectively is not pumped; and (*4*) electron leak in the univalent reduction of oxygen (O_2; dioxygen) to superoxide anion radical ($O_2^{\bullet-}$).

could this P be confused with phosphate or phosphorylation?

Box 3: Coupling, power and efficiency, at constant temperature and pressure

Energetic coupling means that two processes of energy transformation are linked such that the input power, P_{in}, is the driving element of the output power, P_{out}, and the out/input power ratio is the efficiency. In general, power is work per unit time [$J.s^{-1}$=W]. When describing a system with volume V without information on the internal structure, the output is defined as the *external*

~~same~~ another P?

457 work (exergy) performed by the *total* system on its environment. Such as system may be open
458 for any type of exchange, or closed and thus allowing only heat and work to be exchanged
459 across the system boundaries. This is the classical black box approach of thermodynamics. In
460 contrast, in a colourful compartmental analysis of *internal* energy transformations (**Fig. 2**), the
461 system is structured and described by definition of internal compartments (with information on
462 the heterogeneity of the system; **Box 2**) and analysis of separate parts, *i.e.* a sequence of *partial*
463 energy transformations, tr. In general, power per unit volume, P_{tr}/V [W.L^{-1}], is the product of a
464 volume-specific flux, J_{tr}, and its conjugated force, F_{tr}, and is closely linked to the dissipation
465 function using the terminology of irreversible thermodynamics (Prigogine 1967; Gnaiger
466 1993a,b). Output power of proton translocation and catabolic input power are (**Fig. 2**),

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

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

469 $F_{O2,k}$ is the exergonic input force with a negative sign, and, $F_{H+,out}$, is the endergonic output
470 force with a positive sign (**Box 4**). Ergodynamic efficiency is the ratio of output/input power,
471 or the flux ratio times force ratio (Gnaiger 1993a,b),

472 $$\varepsilon = \frac{P_{H+,out}}{-P_k} = \frac{J_{H+,out}}{J_{O2,k}} \cdot \frac{F_{H+,out}}{-F_{O2,k}}$$

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

fixed ratio technically defined as a stoichiometric relationship (a gear ratio in a mechanical system). Such maximal stoichiometric output/input flux ratios are considered in OXPHOS analysis as the upper limits or mechanistic H^+_{out}/O_2 and $P\gg/O_2$ ratios (**Fig. 1**).

The steady-state: Mitochondria represent a thermodynamically open system functioning as a biochemical transformation system in non-equilibrium states. State variables (protonmotive force; redox states) and metabolic fluxes (*rates*) are measured in defined mitochondrial respiratory *states*. Strictly, steady states can be obtained only in open systems, in which changes due to *internal* transformations, *e.g.*, O_2 consumption, are instantaneously compensated for by *external* fluxes *e.g.*, O_2 supply, such that oxygen concentration does not change in the system (Gnaiger 1993b). Mitochondrial respiratory states monitored in closed systems satisfy the criteria of pseudo-steady states for limited periods of time, when changes in the system (concentrations of O_2, fuel substrates, ADP, P_i, H^+) do not exert significant effects on metabolic fluxes (respiration, phosphorylation). Such pseudo-steady states require respiratory media with sufficient buffering capacity and kinetically saturating concentrations of substrates to be maintained, and thus depend on the kinetics of the processes under investigation. Proton turnover, $J_{\infty H+}$, and ATP turnover, $J_{\infty P}$, proceed in the steady-state at constant $F_{H+,out}$, when $J_{\infty H+} = J_{H+,out} = J_{H+,in}$, and at constant $F_{P\gg}$, when $J_{\infty P} = J_{P\gg} = J_{\ll P}$ (**Fig. 2**).

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

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

LEAK state (Fig. 3): LEAK state is defined as a state of mitochondrial respiration when O_2 flux mainly compensates for the proton leak in the absence of ATP synthesis, at kinetically saturating concentrations of O_2 and respiratory substrates. LEAK respiration is measured to obtain an indirect estimate of *intrinsic uncoupling* without addition of any experimental uncoupler: (*1*)

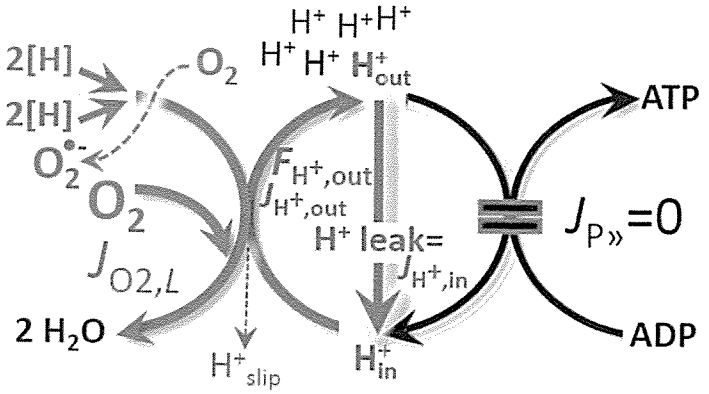


Fig. 3. LEAK state: Phosphorylation is arrested, $J_{P»}$=0, and oxygen flux, $J_{O2,L}$, is controlled mainly by the proton leak, which equals $J_{H+,in}$, at maximum protonmotive force, $F_{H+,out}$ (See also Fig. 2).

524 in the absence of adenylates; (*2*) after depletion of ADP at maximum ATP/ADP ratio; or (*3*)
525 after inhibition of the phosphorylation system by inhibitors of ATP synthase, such as
526 oligomycin, or adenine nucleotide translocase, such as carboxyatractyloside.

527 **Proton leak:** Proton leak is the *uncoupled* process in which protons are translocated
528 across the inner mitochondrial membrane in the dissipative direction of the downhill
529 protonmotive force without coupling to phosphorylation (**Fig. 3**). The proton leak flux depends
530 on the protonmotive force, is a property of the inner mitochondrial membrane, may be enhanced
531 due to possible contaminations by free fatty acids, and is physiologically controlled. In
532 particular, uncoupling mediated by uncoupling protein 1 (UCP1) is physiologically controlled,
533 *e.g.*, in brown adipose tissue. UCP1 is a proton channel of the inner mitochondrial membrane
534 facilitating the conductance of protons across the inner mitochondrial membrane. As
535 consequence of this effective short-circuit, the protonmotive force diminishes, resulting in
536 stimulation of electron transfer to oxygen and heat dissipation without phosphorylation of ADP.
537 Mitochondrial injuries may lead to *dyscoupling* as a pathological or toxicological cause of
538 *uncoupled* respiration, *e.g.*, as a consequence of opening the permeability transition pore.
539 Dyscoupled respiration is distinguished from the experimentally induced *noncoupled*
540 respiration in the ETS state. Under physiological conditions, the proton leak is the dominant
541 contributor to the overall leak current.

542 **Proton slip:** Proton slip is the *decoupled* process in which protons are only partially
543 translocated by a proton pump of the ETS and slip back to the original compartment (Dufour *et*
544 *al.* 1996). Proton slip can also happen in association with the ATP-synthase, in which case the
545 proton slips downhill across the membrane to the matrix without contributing to ATP synthesis.
546 In each case, proton slip is a property of the proton pump and increases with the turnover rate
547 of the pump.

548 **Cation cycling:** Proton leak is a leak current of protons. There can be other cation
549 contributors to leak current including calcium and probably magnesium. Calcium current is

balanced by mitochondrial Na/Ca exchange, which is balanced by Na/H exchange or K/H exchange. This is another effective uncoupling mechanism different from proton leak and slip.

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

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

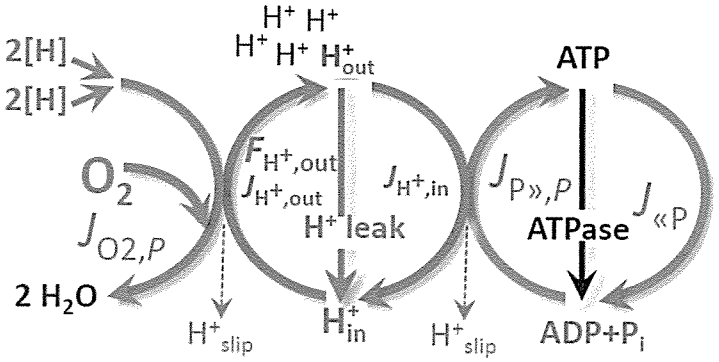


Fig. 4. OXPHOS state: Phosphorylation, $J_{P»}$, is stimulated by kinetically saturating [ADP] and inorganic phosphate, [P_i], and is supported by a high protonmotive force, $F_{H+,out}$. O_2 flux, $J_{O2,P}$, is highly coupled at a maximum P»/O_2 ratio, $J_{P»,P}/J_{O2,P}$ (See also Fig. 2).

Respiratory capacities at kinetically saturating substrate concentrations provide reference values or upper limits of performance, aiming at the generation of data sets for comparative purposes. Any effects of substrate kinetics are thus separated from reporting actual mitochondrial capacity for oxidation during coupled respiration, against which physiological activities can be evaluated.

As discussed previously, 0.2 mM ADP does not fully saturate flux in isolated mitochondria (Gnaiger 2001; Puchowicz *et al.* 2004); a greater ADP concentration is required, particularly in permeabilized muscle fibres and cardiomyocytes, to overcome limitations by intracellular diffusion and by the reduced conductance of the outer mitochondrial membrane (Jepihhina *et al.* 2011, Illaste *et al.* 2012, Simson *et al.* 2016) either through interaction with tubulin (Rostovtseva *et al.* 2008) or other intracellular structures (Birkedal *et al.* 2014). In

permeabilized muscle fibre bundles of high respiratory capacity, the apparent K_m for ADP increases up to 0.5 mM (Saks *et al.* 1998), indicating that >90% saturation is reached only at >5 mM ADP. Similar ADP concentrations are also required for accurate determination of OXPHOS capacity in human clinical cancer samples and permeabilized cells (ref).

Table 2. Distinction of terms related to coupling.

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

ETS state (Fig. 5): The ETS state is defined as the *noncoupled* state with kinetically saturating concentrations of O_2, respiratory substrate and optimum *exogenous* uncoupler concentration for maximum O_2 flux, as an estimate of oxidative ETS capacity. Inhibition of respiration is observed at higher than optimum uncoupler concentrations. As a consequence of the nearly collapsed protonmotive force, the driving force is insufficient for phosphorylation and $J_{P»}=0$.

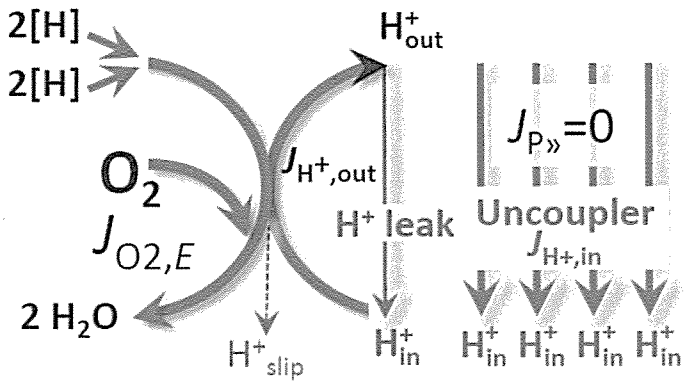


Fig. 5. ETS state: Noncoupled respiration, $J_{O2,E}$, is maximum at optimum exogenous uncoupler concentration and phosphorylation is zero, $J_{P»}=0$ (See also Fig. 2).

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

597 **ROX:** Residual oxygen consumption (ROX) is defined as O_2 consumption due to
598 oxidative side reactions remaining after inhibition of the ETS. ROX is not a coupling state but
599 represents a baseline that is used to correct mitochondrial respiration in defined coupling states.
600 ROX is not necessarily equivalent to non-mitochondrial respiration, considering oxygen-
601 consuming reactions in mitochondria not related to ETS, such as oxygen consumption in
602 reactions catalyzed by monoamine oxidases (type A and B), monooxygenases (cytochrome
603 P450 monooxygenases), dioxygenase (sulfur dioxygenase and trimethyllysine dioxygenase),
604 several hydoxylases, and more. Mitochondrial preparations, especially those obtained from
605 liver, are contaminated by peroxisomes. This fact makes the exact determination of
606 mitochondrial oxygen consumption and mitochondria-associated generation of reactive oxygen
607 species complicated (Schönfeld *et al.* 2009). The dependence of ROX-linked oxygen
608 consumption needs to be studied in detail with respect to non-ETS enzyme activities,
609 availability of specific substrates, oxygen concentration, and electron leakage leading to the
610 formation of reactive oxygen species.

611

612 *2.3. Coupling states and respiratory rates*

613 It is important to distinguish metabolic systems or modules from metabolic states and the
614 corresponding metabolic rates; for example: electron transfer system, ETS (**Fig. 6**), ETS state
615 (**Fig. 5**), and ETS capacity, *E*, respectively (**Table 1**). The protonmotive force is *high* in the
616 OXPHOS state when it drives phosphorylation, *maximum* in the LEAK state of coupled
617 mitochondria, driven by LEAK respiration at a minimum back flux of protons to the matrix
618 side, and *very low* in the ETS state when uncouplers short-circuit the proton cycle (**Table 1**).

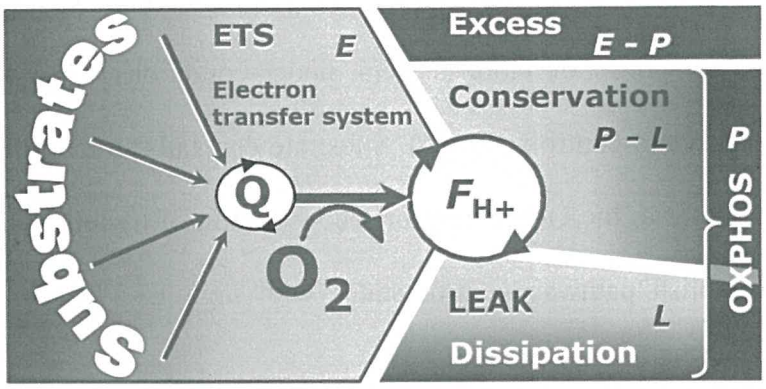


Fig. 6. Four-compartment model of oxidative phosphorylation. Respiratory states (ETS, OXPHOS, LEAK) and corresponding rates (*E*, *P*, *L*) are connected by the protonmotive force, $F_{H+,out}$. Electron transfer system capacity, *E*, is partitioned into the dissipative LEAK respiration, *L*, partial conservation of the protonmotive exergy (**Box 4**) as the phosphorylation exergy in net OXPHOS capacity, *P-L*, and the excess capacity, *E-P*. Modified from Gnaiger (2014).

The three coupling states, ETS, LEAK and OXPHOS, are presented in a schematic context with the corresponding respiratory rates, abbreviated as *E*, *L* and *P*, respectively (**Fig. 6**). This clarifies that *E* may exceed or be equal to *P*, but *E* cannot theoretically be lower than *P*. *E*<*P* must be discounted as an artefact, which may be caused experimentally by: (*1*) loss of oxidative capacity during the time course of the respirometric assay, since *E* is measured subsequently to *P*; (*2*) using too low uncoupler concentrations; (*3*) using high uncoupler concentrations which inhibit the ETS (Gnaiger 2008); (*4*) high oligomycin concentrations applied for measurement of *L* before titrations of uncoupler, when oligomycin exerts an inhibitory effect on *E*. On the other hand, the excess ETS capacity is overestimated if non-saturating [P_i] or [ADP] (State 3) are used.

is this the first mention of 'state 3'? maybe this (and the other 'Chance' states need to be described first?

E>*P* is observed in many types of mitochondria, varying between species, tissues and cell types. It is the excess ETS capacity pushing the phosphorylation system (**Fig. 1B**) to the limit of its *capacity of utilizing* the protonmotive force. Within any type of mitochondria, the magnitude of *E*>*P* depends on (*1*) the pathway control state with single or multiple electron input into the Q-junction and involvement of three or fewer coupling sites determining the H^+_{out}/O_2 *coupling stoichiometry* (**Fig. 1A**); and (*2*) the *biochemical coupling efficiency*

647 expressed as *(E-L)/E*, since an increase of *L* causes *P* to increase towards the limit of *E*. The
648 *excess E-P* capacity, *E-P*, therefore, provides a sensitive diagnostic indicator of specific injuries
649 of the phosphorylation system, under conditions when *E* remains constant but *P* declines
650 relative to controls (**Fig. 6**). Substrate cocktails supporting simultaneous convergent electron
651 transfer to the Q-junction for reconstitution of tricarboxylic acid cycle (TCA cycle) function
652 establish pathway control states with high ETS capacity, and consequently increase the
653 sensitivity of the *E-P* assay.

654 When subtracting *L* from *P*, the dissipative LEAK component in the OXPHOS state may
655 be overestimated. This can be avoided by measuring LEAK respiration in a state when the
656 protonmotive force is adjusted to its slightly lower value in the OXPHOS state, *e.g.*, by titration
657 of an ETS inhibitor. Any turnover-dependent components of proton leak and slip, however, are
658 underestimated under these conditions (Garlid *et al.* 1993). In general, it is inappropriate to use
659 the term *ATP production* for the difference of oxygen consumption measured in states *P* and *L*.
660 The difference *P-L* is the upper limit of the part of OXPHOS capacity that is freely available
661 for ATP production (corrected for LEAK respiration) and is fully coupled to phosphorylation
662 with a maximum mechanistic stoichiometry (**Fig. 6**).
663

664 *2.4. Classical terminology for isolated mitochondria*

665 *'When a code is familiar enough, it ceases appearing like a code; one forgets that*
666 *there is a decoding mechanism. The message is identical with its meaning'*
667 (Hofstadter 1979).

668 Chance and Williams (1955; 1956) introduced five classical states of mitochondrial respiration
669 and cytochrome redox states. **Table 3** shows a protocol with isolated mitochondria in a closed
670 respirometric chamber, defining a sequence of respiratory states.
671

672 **Table 3. Metabolic states of mitochondria (Chance and**
673 **Williams, 1956; Table V).**
674

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

675

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

679 **State 2** is induced by addition of a high concentration of ADP (typically 100 to 300 μM),
680 which stimulates respiration transiently on the basis of endogenous fuel substrates and
681 phosphorylates only a small portion of the added ADP. State 2 is then obtained at a low
682 respiratory activity limited by zero endogenous fuel substrate availability (**Table 3**). If addition
683 of specific inhibitors of respiratory complexes, such as rotenone, does not cause a further
684 decline of oxygen consumption, State 2 is equivalent to residual oxygen consumption (See
685 below). If inhibition is observed, undefined endogenous fuel substrates are a confounding factor
686 of pathway control by externally added substrates and inhibitors. In contrast to the original
687 definition, an alternative protocol is frequently applied, in which State 2 is induced by addition
688 of fuel substrate without ADP (LEAK state), followed by addition of ADP.

689 **State 3** is the state stimulated by addition of fuel substrates while the ADP concentration
690 is still high (**Table 3**) and supports coupled energy transformation through oxidative
691 phosphorylation. 'High ADP' is a concentration of ADP specifically selected to allow the
692 measurement of State 3 to State 4 transitions of isolated mitochondria in a closed respirometric
693 system. Repeated ADP titration re-establishes State 3 at 'high ADP'. Starting at oxygen
694 concentrations near air-saturation (ca. 200 μM O_2 at sea level and 37 °C), the total ADP
695 concentration added must be low enough (typically 100 to 300 μM) to allow phosphorylation

696 to ATP at a coupled oxygen consumption that does not lead to oxygen depletion during the
697 transition to State 4. In contrast, kinetically saturating ADP concentrations usually are an order
698 of magnitude higher than 'high ADP', *e.g.* 2.5 mM in isolated mitochondria. The abbreviation
699 State 3u is frequently used in bioenergetics, to indicate the state of respiration after titration of
700 an uncoupler, without sufficient emphasis on the fundamental difference between OXPHOS
701 capacity (*well-coupled* with an *endogenous* uncoupled component) and ETS capacity
702 (*noncoupled*).

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

715 **State 5** is the state after exhaustion of oxygen in a closed respirometric chamber.
716 Diffusion of oxygen from the surroundings into the aqueous solution may be a confounding
717 factor preventing complete anoxia (Gnaiger 2001).

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

721

3. States and rates

3.1. The protonmotive force and proton flux

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

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

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

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

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

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

2: The protonmotive force is $F_{H+,out}$, expressed either in isomorphic format *e* or *n*. $F_{el/e} \equiv \Delta\Psi$ is the partial protonmotive force (el) acting generally on charged motive molecules (*i.e.* ions that are displaceable across the inner mitochondrial membrane). In contrast, $F_{H+,d/n} \equiv \Delta\mu_{H+}$ is the partial protonmotive force specific for proton displacement (H^+_d). The sign of the force is negative for exergonic transformations in which exergy is lost or dissipated, and positive for endergonic transformations which conserve exergy from a coupled exergonic process (**Box 4**).

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

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

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

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

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

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

768 **Chemical part of the protonmotive force:** (*1*) Isomorph *n:* $F_{d,H+/n} \equiv \Delta\mu_{H+}$ is the chemical
769 part (diffusion, displacement of H^+) of the protonmotive force expressed in units joule per mole
770 [J/mol]. $F_{d,H+/n}$ is defined as partial Gibbs energy change per *motive amount of protons, n* [mol]
771 (**Table 4**, Note 2*n*). (*2*) Isomorph *e*: $F_{d,H+/e} \equiv \Delta\mu_{H+}/F$ is the chemical force expressed in units
772 joule per coulomb [V], defined as partial Gibbs energy change per *motive amount of protons*
773 *expressed in units of electric charge, e* [C], but specific for proton charge (**Table 4**, Note 2*e*).
774 Protonmotive means that there is a potential for the movement of protons, and force is a
775 measure of the potential for motion. Motion is relative and not absolute (Principle of Galilean
776 Relativity); likewise there is no absolute potential, but (isomorphic) forces are potential
777 differences. An electric partial force expressed in the format of electric charge, $F_{el/e}$, of -0.2 V
778 (**Table 5**, Note 5*e*) is equivalent to force in the format of amount, $F_{el,H+/n}$, of 19 $kJ \cdot mol^{-1}$ H^+_{out}
779 (Note 5*n*). For a ΔpH of 1 unit, the chemical partial force in the format of amount, $F_{d,H+/n}$,
780 changes by 5.9 $kJ \cdot mol^{-1}$ (**Table 5**, Note 6*n*) and chemical force in the format of charge $F_{d,H+/e}$
781 changes by 0.06 V (Note 6*e*). Considering a driving force of -470 $kJ \cdot mol^{-1}$ O_2 for oxidation, the
782 thermodynamic limit of the H^+_{out}/O_2 ratio is reached at a value of 470/19=24, compared to a
783 mechanistic stoichiometry of 20 (**Fig. 1**).
784

---

785 **Box 4: Endergonic and exergonic transformations, exergy and dissipation**

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

793 In contrast, energy cannot be lost or produced in any internal process, which is the key
794 message of the first law of thermodynamics. Thus mitochondria are the sites of energy
795 transformation but not energy production. Open and closed systems can gain energy and exergy
796 only by external fluxes, *i.e.* uptake from the environment. Exergy is the potential to perform
797 work. In the framework of flux-force relationships (**Box 3**), the *partial* derivative of Gibbs
798 energy per advancement of a transformation is an isomorphic force, F_{tr} (**Table 5**, Note 2). In
799 other words, force is equal to exergy/motive unit (in integral form, this definition takes care of
800 non-isothermal processes). This formal generalization represents an appreciation of the
801 conceptual beauty of Peter Mitchell's innovation of the protonmotive force against the
802 background of the established paradigm of the electromotive force (emf) defined at the limit of
803 zero current (Cohen *et al.* 2008).

804

805 **Table 5. Power, exergy, force, flux, and advancement.**
806

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

807
808 1 to 4: An isomorphic motive entity or transformant, expressed in units x, is defined for any
809 transformation, tr. x=mol or C in proton translocation.

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

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

4*n*: For a chemical reaction, the advancement of reaction r is $d_r\xi_B=d_rn_B\cdot\nu_B^{-1}$ [mol]. The stoichiometric number is $\nu_B=-1$ or $\nu_B=1$, depending on B being a product or substrate, respectively, in reaction r involving one mole of B. The conjugated *intensive* molar quantity, $F_{B,r} = \partial_rG/\partial_r\xi_B$ [J·mol^{-1}], is the chemical force of reaction or *reaction-motive* force per stoichiometric amount of B. In reaction kinetics, d_rn_B is expressed as a volume-specific quantity, which is the partial contribution to the total concentration change of B, $d_rc_B=d_rn_B/V$ and $dc_B=dn_B/V$, respectively. In open systems with constant volume V, $dc_B=d_rc_B+d_ec_B$, where r indicates the *internal* reaction and e indicates the *external* flux of B into the unit volume of the system. At steady state the concentration does not change, $dc_B=0$, when d_rc_B is compensated for by the external flux of B, $d_rc_B=-d_ec_B$ (Gnaiger 1993b). Alternatively, $dc_B=0$ when B is held constant by different coupled reactions in which B acts as a substrate or a product.

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

5*n*: F=96.5 (kJ·mol^{-1})/V.

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

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

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

3.2. Forces and fluxes in physics and irreversible thermodynamics

According to its definition in physics, a potential difference and as such the *protonmotive force*, Δp_{H+}, is not a force *per se* (Cohen *et al.* 2008). The fundamental forces of physics are distinguished from *motive forces* of statistical and irreversible thermodynamics. Complementary to the attempt towards unification of fundamental forces defined in physics,

840 the concepts of Nobel laureates Lars Onsager, Erwin Schrödinger, Ilya Prigogine and Peter
841 Mitchell (even if expressed in apparently unrelated terms) unite the diversity of *generalized* or
842 'isomorphic' *flux-force* relationships, the product of which links to the dissipation function and
843 Second Law of thermodynamics (Schrödinger 1944; Prigogine 1967). A *motive force* is the
844 derivative of potentially available or 'free' energy (exergy) per isomorphic *motive* unit (Box 4).
845 Perhaps the first account of a *motive force* in energy transformation can be traced back to the
846 Peripatetic school around 300 BC in the context of moving a lever, up to Newton's motive force
847 proportional to the alteration of motion (Coopersmith 2010).

848 **Vectorial and scalar forces, and fluxes:** In chemical reactions and osmotic or diffusion
849 processes occurring in a closed heterogeneous system, such as a chamber containing isolated
850 mitochondria, scalar transformations occur without measured spatial direction but between
851 separate compartments (translocation between the matrix and intermembrane space) or between
852 energetically-separated chemical substances (reactions from substrates to products). Hence, the
853 corresponding fluxes are not vectorial but scalar, and are expressed per volume and not per
854 membrane area (Box 2). The corresponding motive forces are also scalar potential *differences*
855 across the membrane (**Table 5**), without taking into account the *gradients* across the 6 nm thick
856 inner mitochondrial membrane (Rich 2003).

857 **Coupling:** In energetics (ergodynamics), coupling is defined as an exergy transformation
858 fuelled by an exergonic (downhill) input process driving the advancement of an endergonic
859 (uphill) output process. The (negative) output/input power ratio is the efficiency of a coupled
860 energy transformation (Box 3). At the limit of maximum efficiency of a completely coupled
861 system, the (negative) input power equals the (positive) output power, such that the total power
862 approaches zero at the maximum efficiency of 1, and the process becomes fully reversible
863 without any dissipation of exergy, i.e. without entropy production.

864 **Coupled versus bound processes:** Since the chemiosmotic theory describes the
865 mechanisms of coupling in OXPHOS, it may be interesting to ask if the electrical and chemical

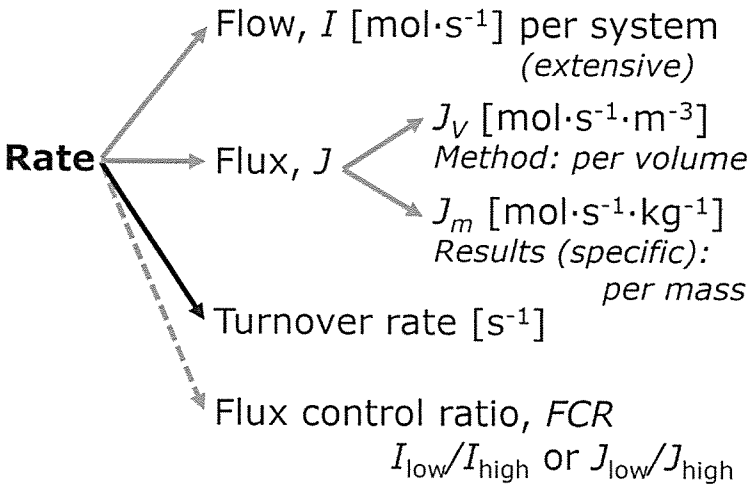
parts of proton translocation are coupled processes. This is not the case according to the definition of coupling. If the coupling mechanism is disengaged, the output process becomes independent of the input process, and both proceed in their downhill (exergonic) direction (**Fig. 2**). It is not possible to physically uncouple the electrical and chemical processes, which are only *theoretically* partitioned as electrical and chemical components and can be measured separately. If partial processes are non-separable, *i.e.*, cannot be uncoupled, then these are not *coupled* but are defined as *bound* processes. The electrical and chemical parts are tightly bound partial forces of the protonmotive force, since a flux cannot be partitioned but expressed only in either an electrical or chemical isomorphic format (**Table 4**).

4. Normalization: fluxes and flows

4.1. Flux per chamber volume

The volume-specific *flux of a chemical reaction* r is the time derivative of the advancement of the reaction per unit volume, $J_{V,B} = d_r\xi_B/dt \cdot V^{-1}$ [(mol·s^{-1})·L^{-1}]. The *rate of concentration change* is dc_B/dt [(mol·L^{-1})·s^{-1}], where concentration is $c_B=n_B/V$. It is helpful to make the subtle distinction between [mol·s^{-1}·L^{-1}] and [mol·L^{-1}·s^{-1}] for the fundamentally different quantities of volume-specific flux and rate of concentration change, which merge to a single expression only in closed systems. In open systems, external fluxes (such as O_2 supply) are distinguished from internal transformations (metabolic flux, O_2 consumption). In a closed system, external flows of all substances are zero and O_2 consumption (internal flow), I_{O2} [pmol·s^{-1}], causes a decline of the amount of O_2 in the system, n_{O2} [nmol]. Normalization of these quantities for the volume of the system, V [L=dm^3], yields volume-specific O_2 flux, $J_{V,O2}=I_{O2}/V$ [nmol·s^{-1}·L^{-1}], and O_2 concentration, [O_2] or $c_{O2}=n_{O2}/V$ [nmol·mL^{-1}=µmol·L^{-1}=µM]. Instrumental background O_2 flux is due to external flux into a non-ideal closed respirometer, such that total volume-specific flux has to be corrected for instrumental background O_2 flux, i.e. O_2 diffusion into or out of the instrumental chamber. $J_{V,O2}$ is relevant mainly for

892 methodological reasons and should be compared with the accuracy of instrumental resolution
893 of background-corrected flux, *e.g.* ±1 nmol·s^{-1}·L^{-1} (Gnaiger 2001). 'Metabolic' or catabolic
894 indicates O_2 flux, $J_{O2,k}$, corrected for instrumental background O_2 flux and chemical background
895 O_2 flux due to autoxidation of chemical components added to the incubation medium.
896
897 *4.2. Extensive quantities and size-specific normalization*
898 Application of common and generally defined units is required for direct transfer of
899 reported results into a database. The second [s] is the *SI* unit for the base quantity *time*. It is also
900 the standard time-unit used in solution chemical kinetics. **Table 6** lists some conversion factors
901 to obtain *SI* units. The term *rate* is not sufficiently defined to be useful for a database (**Fig. 7**).
902 The inconsistency of the meanings of rate becomes fully apparent when considering Galileo
903 Galilei's famous principle, that 'bodies of different weight all fall at the same rate (have a
904 constant acceleration)' (Coopersmith 2010).
905 **Extensive quantities:** An extensive quantity increases proportionally with system size.
906 The magnitude of an extensive quantity is completely additive for non-interacting subsystems,
907 such as mass or flow expressed per defined system. The magnitude of these quantities depends
908 on the extent or size of the system (Cohen *et al.* 2008).
909



910 **Fig. 7. Different meanings of *rate* may lead to confusion, if the normalization is not sufficiently**
911 **specified.** Results are frequently expressed as mass-specific *flux*, J_m, per mg protein, dry or wet weight
912 (mass). Cell volume, V_{cell}, or mitochondrial volume, V_{mt}, may be used for normalization (volume-

919 specific flux, J_{Vcell} or J_{Vmt}), which then must be clearly distinguished from flux, J_V, expressed for
920 methodological reasons per volume of the measurement system, or flow per cell, I_X.
921
922 **Size-specific quantities:** 'The adjective *specific* before the name of an extensive quantity
923 is often used to mean *divided by mass*' (Cohen *et al.* 2008). Mass-specific flux is flow divided
924 by mass of the system. A mass-specific quantity is independent of the extent of non-interacting
925 homogenous subsystems. Tissue-specific quantities are of fundamental interest in comparative
926 mitochondrial physiology, where *specific* refers to the *type* rather than *mass* of the tissue. The
927 term *specific*, therefore, must be further clarified, such that tissue mass-specific, *e.g.*, muscle
928 mass-specific quantities are defined.
929 **Molar quantities:** 'The adjective *molar* before the name of an extensive quantity
930 generally means *divided by amount of substance*' (Cohen *et al.* 2008). The notion that all molar
931 quantities then become *intensive* causes ambiguity in the meaning of *molar Gibbs energy*. It is
932 important to emphasize the fundamental difference between normalization for amount of
933 substance *in a system* or for amount of motive substance *in a transformation*. When the Gibbs
934 energy of a system, G [J], is divided by the amount of substance B in the system, n_B [mol], a
935 *size-specific* molar quantity is obtained, $G_B = G/n_B$ [J·mol^{-1}], which is not any force at all. In
936 contrast, when the partial Gibbs energy change, $\partial_r G$ [J], is divided by the motive amount of
937 substance B in reaction r (advancement of reaction), $\partial_r \xi_B$ [mol], the resulting intensive molar
938 quantity, $F_{r,B} = \partial G/\partial_r \xi_B$ [J·mol^{-1}], is the chemical motive force of reaction r involving 1 mol B
939 (**Table 5**, Note 4).
940 **Flow per system, *I*:** In analogy to electrical terms, flow as an extensive quantity (I; per
941 system) is distinguished from flux as a size-specific quantity (J; per system size) (**Fig. 7**).
942 Electric current is flow, I_{el} [A=C·s^{-1}] per system (extensive quantity). When dividing this
943 extensive quantity by system size (membrane area), a size-specific quantity is obtained, which
944 is electric flux (electric current density), $\boldsymbol{J}_{el}$ [A·m^{-2} = C·s^{-1}·m^{-2}].

945 **Size-specific flux, *J***: Metabolic O_2 flow per tissue increases as tissue mass is increased.
946 Tissue mass-specific O_2 flux should be independent of the size of the tissue sample studied in
947 the instrument chamber, but volume-specific O_2 flux (per volume of the instrument chamber,
948 V) should increase in direct proportion to the amount of sample in the chamber. Accurate
949 definition of the experimental system is decisive: whether the experimental chamber is the
950 closed, open, isothermal or non-isothermal *system* with defined volume as part of the
951 measurement apparatus, in contrast to the experimental *sample* in the chamber (**Table 6**).
952 Volume-specific O_2 flux depends on mass-concentration of the sample in the chamber, but
953 should be independent of the chamber volume. There are practical limitations to increasing the
954 mass-concentration of the sample in the chamber, when one is concerned about crowding
955 effects and instrumental time resolution.

956 **Sample concentration C_{mX}:** Normalization for sample concentration is required for
957 reporting respiratory data. Consider a tissue or cells as the sample, X, and the sample mass, m_X
958 [mg] from which a mitochondrial preparation is obtained. The sample mass is frequently
959 measured as wet or dry weight ($m_X \equiv W_w$ or W_d [mg]), or as amount of tissue or cell protein
960 ($m_X = m_{Protein}$). In the case of permeabilized tissues, cells, and homogenates, the sample
961 concentration, $C_{mX} = m_X/V$ [$mg \cdot mL^{-1} = g \cdot L^{-1}$], is simply the mass of the subsample of tissue that is
962 transferred into the instrument chamber. Part of the mitochondria from the tissue is lost during
963 preparation of isolated mitochondria, and only a fraction of mitochondria is obtained, expressed
964 as the mitochondrial yield (**Fig. 8**). At a high mitochondrial yield the sample of isolated
965 mitochondria is more representative of the total mitochondrial population than in preparations
966 characterized by low mitochondrial yield. Determination of the mitochondrial yield is based on
967 measurement of the concentration of a mitochondrial marker in the tissue homogenate, $C_{mte,thom}$,
968 which simultaneously provides information on the specific mitochondrial density in the sample
969 (**Fig. 8**).

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

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

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

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

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

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

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

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

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

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

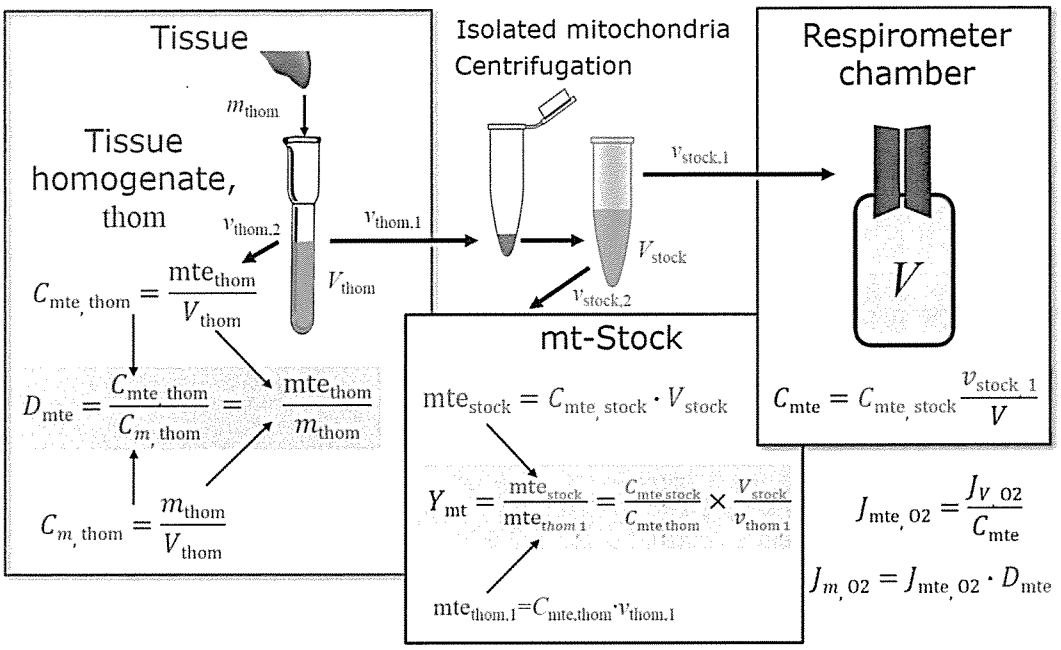
970 Tissues can contain multiple cell populations which may have distinct mitochondrial
971 subtypes. Mitochondria are also in a constant state of flux due to highly dynamic fission and
972 fusion cycles, and can exist in multiple stages and sizes which may be altered by a range of
973 factors. The isolation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

980

981 **Table 6. Sample concentrations and normalization of flux with *SI* base units.**
982

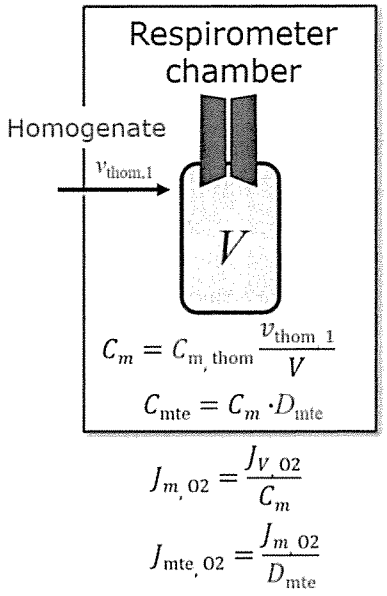
Expression	Symbol	Definition	*SI* Unit	Notes
Sample				
Identity of sample	X	Cells, animals, patients		
Number of sample entities X	N_X	Number of cells, *etc.*	x	
Mass of sample X	m_X		kg	1
Mass of entity X	M_X	$M_X = m_X \cdot N_X^{-1}$	$kg \cdot x^{-1}$	1
Mitochondria				
Mitochondria	mt	X=mt		
Amount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

983



1014

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



1015

1016 **Fig. 8. Normalization of volume-specific flux of isolated mitochondria and tissue**
1017 **homogenate. A:** Mitochondrial yield, Y_{mt}, in preparation of isolated mitochondria. $v_{thom,1}$
1018 and $v_{stock,1}$ are the volumes transferred from the total volume, V_{thom} and V_{stock}, respectively.
1019 $mte_{thom,1}$ is the amount of mitochondrial elements in volume $v_{thom,1}$ used for isolation. **B:**
1020 In respirometry with homogenate, $v_{thom,1}$ is transferred directly into the respirometer
1021 chamber. See **Table 6** for further explanation of symbols.

1022

1023

1024

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

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

Mass-specific flux, $J_{mX,O2}$: Mass-specific flux is obtained by expressing respiration per mass of sample, m_X [mg]. *X* is the type of sample, *e.g.*, tissue homogenate, permeabilized fibres or cells. Volume-specific flux is divided by mass concentration of *X*, $J_{mX,O2} = J_{V,O2}/C_{mX}$; or flow per cell is divided by mass per cell, $J_{mcell,O2} = I_{cell,O2}/M_{cell}$. If mass-specific O_2 flux is constant and independent of sample size (expressed as mass), then there is no interaction between the subsystems. A 1.5 mg and a 3.0 mg muscle sample respires at identical mass-specific flux. Mass-specific O_2 flux, however, may change with the mass of a tissue sample, cells or isolated mitochondria in the measuring chamber, in which case the nature of the interaction becomes an issue. Optimization of cell density and arrangement is generally important and particularly in experiments carried out in wells, considering the confluency of the cell monolayer or clumps of cells (Salabei *et al.* 2014).

Number concentration, C_{NX}: The experimental *number concentration* of sample in the case of cells or animals, *e.g.*, nematodes is $C_{NX}=N_X/V$ [x·mL^{-1}], where N_X is the number of cells or organisms in the chamber (**Table 6**).

1053 **Flow per sample entity, $I_{X,O2}$:** A special case of normalization is encountered in
1054 respiratory studies with permeabilized (or intact) cells. If respiration is expressed per cell, the
1055 O_2 flow per measurement system is replaced by the O_2 flow per cell, $I_{cell,O2}$ (**Table 6**). O_2 flow
1056 can be calculated from volume-specific O_2 flux, $J_{V,O2}$ [nmol·s^{-1}·L^{-1}] (per V of the measurement
1057 chamber [L]), divided by the number concentration of cells, $C_{Nce}=N_{ce}/V$ [cell·L^{-1}], where N_{ce} is
1058 the number of cells in the chamber. Cellular O_2 flow can be compared between cells of identical
1059 size. To take into account changes and differences in cell size, further normalization is required
1060 to obtain cell size-specific or mitochondrial marker-specific O_2 flux (Renner *et al.* 2003).
1061 The complexity changes when the sample is a whole organism studied as an experimental
1062 model. The well-established scaling law in respiratory physiology reveals a strong interaction
1063 of O_2 consumption and individual body mass of an organism, since *basal* metabolic rate (flow)
1064 does not increase linearly with body mass, whereas *maximum* mass-specific O_2 flux, $\dot{V}_{O2max}$ or
1065 $\dot{V}_{O2peak}$, is approximately constant across a large range of individual body mass (Weibel and
1066 Hoppeler 2005), with individuals, breeds, and certain species deviating substantially from this
1067 general relationship. $\dot{V}_{O2peak}$ of human endurance athletes is 60 to 80 mL O_2·min^{-1}·kg^{-1} body
1068 mass, converted to $J_{m,O2peak}$ of 45 to 60 nmol·s^{-1}·g^{-1} (Gnaiger 2014; **Table 8**).
1069
1070 *4.2. Normalization for mitochondrial content*
1071 Normalization is a problematic subject and it is essential to consider the question of the
1072 study. If the study aims to compare tissue performance, such as the effects of a certain treatment
1073 on a specific tissue, then normalization can be successful, using tissue mass or protein content,
1074 for example. If the aim, however, is to find differences of mitochondrial function independent
1075 of mitochondrial density (**Table 6**), then normalization to a mitochondrial marker is imperative.
1076 However, one cannot assume that quantitative changes in various markers such as
1077 mitochondrial proteins necessarily occur in parallel with one another. It is important to first
1078 establish that the marker chosen is not selectively altered by the performed treatment. In

conclusion, the normalization must reflect the question under investigation to reach a satisfying answer. On the other hand, the goal of comparing results across projects and institutions requires some standardization on normalization for entry into a databank.

Mitochondrial concentration, C_{mte}, and mitochondrial markers: It is important that mitochondrial content in the tissue and the measurement chamber be quantified, as a physiological output and result of mitochondrial biogenesis and degradation, and as a quantity for normalization in functional analyses. Mitochondrial organelles comprise a cellular reticulum that is in a continual flux of fusion and fission. Hence the definition of an "amount" of mitochondria is often misconceived: mitochondria cannot be counted as a number of occurring elements. Therefore, quantification of the "amount" of mitochondria depends on measurement of chosen mitochondrial markers. 'Mitochondria are the structural and functional elemental units of cell respiration' (Gnaiger 2014). The quantity of a mitochondrial marker can be considered as the measurement of the amount of *elemental mitochondrial units* or *mitochondrial elements*, mte. However, since mitochondrial quality changes under certain stimuli, particularly in mitochondrial dysfunction, some markers can vary while other markers are unchanged. (*1*) Mitochondrial volume or membrane area are structural markers, whereas mitochondrial protein mass is frequently used as a marker for isolated mitochondria. (*2*) Mitochondrial marker enzymes (amounts or activities) and molecular markers can be selected as matrix markers, *e.g.*, citrate synthase activity, mtDNA; or inner mt-membrane markers, *e.g.*, cytochrome *c* oxidase activity, aa_3 content, cardiolipin, TOM20. (*3*) Extending the measurement of mitochondrial marker enzyme activity to mitochondrial pathway capacity, measured as ETS or OXPHOS capacity, can be considered as an integrative functional mitochondrial marker.

Depending on the type of mitochondrial marker, the mitochondrial elements, mte, are expressed in marker-specific units. Although concentration and density are used synonymously in physical chemistry, it is recommended to distinguish *experimental mitochondrial*

concentration, C_{mte}=mte/V and *physiological mitochondrial density*, D_{mte}= mte/m_X. Then mitochondrial density is the amount of mitochondrial elements per mass of tissue. The former is mitochondrial density multiplied by sample mass concentration, $C_{mte}=D_{mte}\cdot C_{mX}$, or mitochondrial content multiplied by sample number concentration, $C_{mte}=mte_X\cdot C_{NX}$ (**Table 6**).

Mitochondria-specific flux, $J_{mte,O2}$: Volume-specific metabolic O_2 flux depends on: (*1*) the sample concentration in the volume of the instrument chamber, C_{mX}, or C_{NX}; (*2*) the mitochondrial density in the sample, D_{mte}=mte/m_X or mte_X=mte/N_X; and (*3*) the specific mitochondrial activity or performance per elemental mitochondrial unit, $J_{mte,O2}=J_{V,O2}/C_{mte}$ (**Table 6**). Obviously, the numerical results for $J_{mte,O2}$ vary according to the type of mitochondrial marker chosen for measurement of mte and C_{mte}=mte/V. Some problems are common for all mitochondrial markers: (*1*) Accuracy of measurement is crucial, since even a highly accurate and reproducible measurement of O_2 flux becomes inaccurate and noisy if normalized for a biased and noisy measurement of a mitochondrial marker. This problem is acute in mitochondrial respiration because the denominators used (the mitochondrial marker) are often very small moieties whose accurate and precise determination is difficult. This problem can be avoided when O_2 fluxes measured in substrate-uncoupler-inhibitor titration protocols are normalized for flux in a defined respiratory reference state, which is used as an *internal* marker and yields flux control ratios, *FCR*s (**Fig. 7**). *FCR*s are independent of any *externally* measured markers and, therefore, are statistically very robust. *FCR*s indicate qualitative changes of mitochondrial respiratory control, with highest quantitative resolution, separating the effect of mitochondrial density or concentration on $J_{mX,O2}$ or $I_{X,O2}$ from that of function per elemental mitochondrial marker, $J_{mte,O2}$ (Pesta *et al.* 2011; Gnaiger 2014). (*2*) If mitochondrial quality does not change and only the amount of mitochondria, defined by the chosen mitochondrial marker, varies as a determinant of mass-specific flux, then any marker is equally qualified and selection of the optimum marker depends only on the accuracy and precision of measurement of the mitochondrial marker. (*3*) If mitochondrial flux control ratios

1131 change, then there may not be any best mitochondrial marker. In general, measurement of
1132 multiple mitochondrial markers enables a comparison and evaluation of normalization for a
1133 variety of mitochondrial markers.
1134
1135 *4.3. Conversion: units and normalization*
1136 Many different units have been used to report the rate of oxygen consumption, OCR
1137 (**Table 8**). *SI* base units provide the common reference for introducing the theoretical principles
1138 (**Fig. 7**), and are used with appropriately chosen *SI* prefixes to express numerical data in the
1139 most practical format, with an effort towards unification within specific areas of application
1140 (**Table 9**). For studies of cells, we recommend that respiration be expressed, as far as possible,
1141 as (*1*) O_2 flux normalized for a mitochondrial marker, for separation of the effects of
1142 mitochondrial quality and content on cell respiration (this includes *FCR*s as a normalization for
1143 a functional mitochondrial marker); (*2*) O_2 flux in units of cell volume or mass, for comparison
1144 of respiration of cells with different cell size (Renner *et al.* 2003) and with studies on tissue
1145 preparations, and (*3*) O_2 flow in units of attomole (10^{-18} mol) of O_2 consumed by each cell in a
1146 second [amol·s^{-1}·cell^{-1}], numerically equivalent to [pmol·s^{-1}·10^{-6} cells]. This convention allows
1147 information to be easily used when designing experiments in which oxygen consumption must
1148 be considered. For example, to estimate the volume-specific O_2 flux in an instrument chamber
1149 that would be expected at a particular cell number concentration, one simply needs to multiply
1150 the flow per cell by the number of cells per volume of interest. This provides the amount of O_2
1151 [mol] consumed per time [s^{-1}] per unit volume [L^{-1}]. At an O_2 flow of 100 amol·s^{-1}·cell^{-1} and a
1152 cell density of 10^9 cells·L^{-1} (10^6 cells·mL^{-1}), the volume-specific O_2 flux is 100 nmol·s^{-1}·L^{-1} (100
1153 pmol·s^{-1}·mL^{-1}). Although volume is expressed as m^3 using the *SI* base unit, the litre [dm^3] is the
1154 basic unit of volume for concentration and is used for most solution chemical kinetics. If one
1155 multiplies $I_{cell,O2}$ by C_{Ncell}, then the result will not only be the amount of O_2 [mol] consumed per
1156 time [s^{-1}] in one litre [L^{-1}], but also the change in the concentration of oxygen per second (for

any volume of an ideally closed system). This is ideal for kinetic modeling as it blends with chemical rate equations where concentrations are typically expressed in $mol \cdot L^{-1}$ (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 enucleated platelets.

Table 8. Conversion of various units used in respirometry and ergometry. e is the number of electrons or reducing equivalents. z_B is the charge number of entity B.

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

1 At standard temperature and pressure dry (STPD: 0 °C=273.15 K and 1 atm=101.325 kPa=760 mmHg), the molar volume of an ideal gas, V_m, and $V_{m,O2}$ is 22.414 and 22.392 $L.mol^{-1}$ respectively. Rounded to three decimal places, both values yield the conversion factor of 0.744. For comparison at NTPD (20 °C), $V_{m,O2}$ is 24.038 $L.mol^{-1}$. Note that the *SI* standard pressure is 100 kPa.

1174 2 The multiplication factor is $10^6/(z_B \cdot F)$.

1175 3 The multiplication factor is $z_B \cdot F/10^6$.

1176

1177 **Table 9. Conversion for units with preservation of numerical values.**

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

1178
1179 1 pmol: picomole = 10^{-12} mol
1180 2 amol: attomole = 10^{-18} mol
1181 3 zmol: zeptomole = 10^{-21} mol
1182 4 nmol: nanomole = 10^{-9} mol
1183

1184 *4.4. Conversion: oxygen, proton and ATP flux*

1185 $J_{O2,k}$ is coupled in mitochondrial steady states to proton cycling, $J_{\infty H+} = J_{H+,out} = J_{H+,in}$ (**Fig.**

1186 **2**). $J_{H+,out/n}$ and $J_{H+,in/n}$ [$nmol \cdot s^{-1} \cdot L^{-1}$] are converted into electrical units, $J_{H+,out/e}$

1187 [$mC \cdot s^{-1} \cdot L^{-1} = mA \cdot L^{-1}$] = $J_{H+,out/n}$ [$nmol \cdot s^{-1} \cdot L^{-1}$]$\cdot F$ [$C \cdot mol^{-1}$]$\cdot 10^{-6}$ (**Table 4**). At a $J_{H+,out}/J_{O2,k}$ ratio

1188 or H^+_{out}/O_2 of 20 (H^+_{out}/O=10), a volume-specific O_2 flux of 100 $nmol \cdot s^{-1} \cdot L^{-1}$ would correspond

1189 to a proton flux of 2,000 nmol $H^+_{out} \cdot s^{-1} \cdot L^{-1}$ or volume-specific current of 193 $mA \cdot L^{-1}$.

1190 $$J_{V,H+out/e}\ [mA \cdot L^{-1}] = J_{V,H+out/n} \cdot F \cdot 10^{-6}\ [nmol \cdot s^{-1} \cdot L^{-1} \cdot mC \cdot nmol^{-1}] \quad \text{(Eq. 3.1)}$$

1191 $$J_{V,H+out/e}\ [mA \cdot L^{-1}] = J_{V,O2} \cdot (H^+_{out}/O_2) \cdot F \cdot 10^{-6}\ [mC \cdot s^{-1} \cdot L^{-1} = mA \cdot L^{-1}] \quad \text{(Eq. 3.2)}$$

1192 ETS capacity in various human cell types including HEK 293, primary HUVEC and fibroblasts

1193 ranges from 50 to 180 $amol \cdot s^{-1} \cdot cell^{-1}$, measured in intact cells in the noncoupled state (see

Gnaiger 2014). At 100 amol·s^{-1}·cell^{-1} corrected for ROX (corresponding to a catabolic power of -48 pW·cell^{-1}), the current across the mt-membranes, I_e, approximates 193 pA·cell^{-1} or 0.2 nA per cell. See Rich (2003) for an extension of quantitative bioenergetics from the molecular to the human scale, with a transmembrane proton flux equivalent to 520 A in an adult at a catabolic power of -110 W. Modelling approaches illustrate the link between proton motive force and currents (Willis *et al.* 2016).For NADH- and succinate-linked respiration, the mechanistic P»/O_2 ratio (referring to the full 4 electron reduction of O_2) is calculated at 20/3.7 and 12/3.7, respectively (Eq. 4) equal to 5.4 and 3.3. The classical P»/O ratios (referring to the 2 electron reduction of 0.5 O_2) are 2.7 and 1.6 (Watt *et al.* 2010), in direct agreement with the measured P»/O ratio for succinate of 1.58 ± 0.02 (Gnaiger *et al.* 2000; for detailed reviews see Wikström and Hummer 2012; Sazanov 2015),

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

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

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

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

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

1220 but integrates mitochondrial physiology with energy transformation in the living cell (Gnaiger
1221 1993a).
1222
1223 **5. Conclusions**
1224 MitoEAGLE can serve as a gateway to better diagnose mitochondrial respiratory defects
1225 linked to genetic variation, age-related health risks, sex-specific mitochondrial performance,
1226 lifestyle with its effects on degenerative diseases, and thermal and chemical environment. The
1227 present recommendations on coupling control states and rates, linked to the concept of the
1228 protonmotive force (Part 1) will be extended in a series of reports on pathway control of
1229 mitochondrial respiration, respiratory states in intact cells, and harmonization of experimental
1230 procedures.
1231

1232 **Box 5: Mitochondrial and cell respiration**
1233 Mitochondrial and cell respiration is the process of highly exothermic energy transformation in
1234 which scalar redox reactions are coupled to vectorial ion translocation across a semipermeable
1235 membrane, which separates the small volume of a bacterial cell or mitochondrion from the
1236 larger volume of its surroundings. The electrochemical exergy can be partially conserved in the
1237 phosphorylation of ADP to ATP or in ion pumping, or dissipated in an electrochemical short-
1238 circuit. Respiration is thus clearly distinguished from fermentation as the counterpart of cellular
1239 core energy metabolism. Respiration is separated in mitochondrial preparations from the partial
1240 contribution of fermentative pathways of the intact cell. According to this definition, residual
1241 oxygen consumption, as measured after inhibition of the mitochondrial electron transfer system,
1242 does not belong to the class of catabolic reactions and is, therefore, subtracted from total oxygen
1243 consumption to obtain baseline-corrected respiration.
1244

1245 The optimal choice for expressing mitochondrial and cell respiration (Box 5) as O_2 flow
1246 per biological system, and normalization for specific tissue-markers (volume, mass, protein)
1247 and mitochondrial markers (volume, protein, content, mtDNA, activity of marker enzymes,
1248 respiratory reference state) is guided by the scientific question. Interpretation of the obtained
1249 data depends critically on appropriate normalization, and therefore reporting rates merely as
1250 $nmol \cdot s^{-1}$ is discouraged, since it restricts the analysis to intra-experimental comparison of
1251 relative (qualitative) differences. Expressing O_2 consumption per cell may not be possible when
1252 dealing with tissues. For studies with mitochondrial preparations, we recommend that
1253 normalizations be provided as far as possible: (*1*) on a per cell basis as O_2 flow (a biophysical
1254 normalization); (*2*) per g cell or tissue protein, or per cell or tissue mass as mass-specific O_2
1255 flux (a cellular normalization); and (*3*) per mitochondrial marker as mt-specific flux (a
1256 mitochondrial normalization). With information on cell size and the use of multiple
1257 normalizations, maximum potential information is available (Renner *et al.* 2003; Wagner *et al.*
1258 2011; Gnaiger 2014). When using isolated mitochondria, mitochondrial protein is a frequently
1259 applied mitochondrial marker, the use of which is basically restricted to isolated mitochondria.
1260 Mitochondrial markers, such as citrate synthase activity as an enzymatic matrix marker, provide
1261 a link to the tissue of origin on the basis of calculating the mitochondrial yield, *i.e.*, the fraction
1262 of mitochondrial marker obtained from a unit mass of tissue.
1263
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1268 Austria.
1269
1270

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