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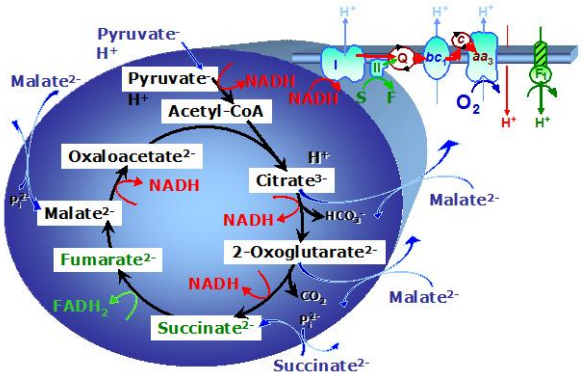
Mitochondrial Pathways through Complexes I+II: Convergent Electron transfer at the Q-Junction and Additive Effect of Substrate Combinations



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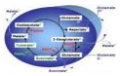
'It is not at all easy to draw a sharp line between cases where what is happening could be called "addition", and where some other word is wanted.'
Douglas R. Hofstadter (1979) Gödel, Escher, Bach: An Eternal Golden Braid. A metaphorical fugue on minds and machines in the spirit of Lewis Carroll. Penguin Books.

Section



Page

1. Electron Transfer System and ET Chain

The term 'electron transfer chain' (or electron transport chain, ETC) is a misnomer. Understanding mitochondrial respiratory control has suffered greatly from this inappropriate terminology, although textbooks using the term ETC (Lehninger 1970; Nicholls & Ferguson 2002) make it sufficiently clear that electron transfer systems are not arranged as a chain: the 'ETC' is in fact not a simple chain but an arrangement of electron transfer complexes in a non-linear, convergent electron transfer system (Hatefi et al 1962; ETS; Fig. 1).

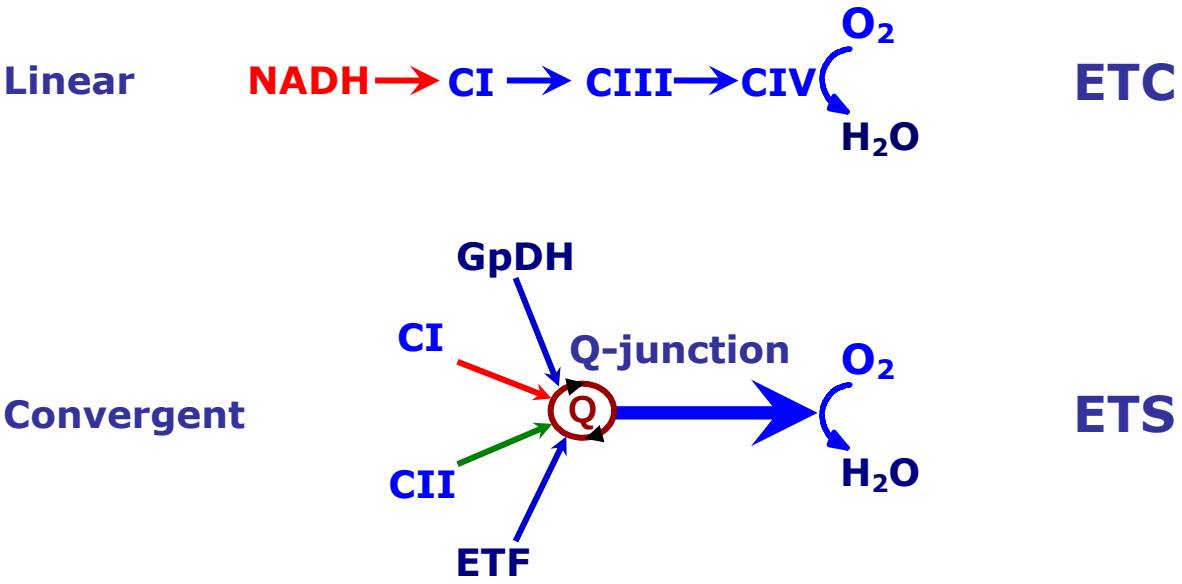


Figure 1. Electron transfer chain (ETC, linear) versus electron transfer system (ETS, convergent). CI to CIV, Complex I to IV; GpDH, glycerophosphate dehydrogenase; ETF, electron transferring flavoprotein.

The firmly established convention of defining the electron transfer chain as being comprised of four Complexes has conceptual weaknesses. (a) In fact, there are at least six Complexes of mitochondrial electron transfer: In addition to Complexes I and II, glycerophosphate dehydrogenase (GpDH) and electron transferring flavoprotein (ETF) are involved in electron transfer to Complex III (Fig. 1). (b) The term 'chain' suggests a linear sequence, whereas the functional structure of the electron transfer system can only be understood by recognizing the convergence of electron flow at the Q-junction, followed by a chain of Complexes III and IV, mediated by cytochrome *c*.

Electrons flow to oxygen from either Complex I with a total of three coupling sites, or from Complex II and other flavoproteins, providing multiple entries into the Q-cycle with two coupling sites downstream. A novel perspective of mitochondrial physiology and respiratory control by simultaneous supply of various substrates emerged from a series of studies based on high-resolution respirometry (OROBOROS Oxygraph-2k; Gnaiger 2001). The design of multiple substrate protocols (Fig. 2; Boushel et al 2007; Aragones et al 2008; Lemieux et al 2011; Pesta et al 2011; Pesta, Gnaiger 2012; Votion et al 2012) is based on a biochemical analysis of convergent pathways comprising the mitochondrial electron transfer system (Fig. 3). When the TCA cycle is in full operation in the intact cell with influx of pyruvate, electron flow into the Q-junction converges according to a NADH: succinate ratio of 4:1 (Fig. 3).

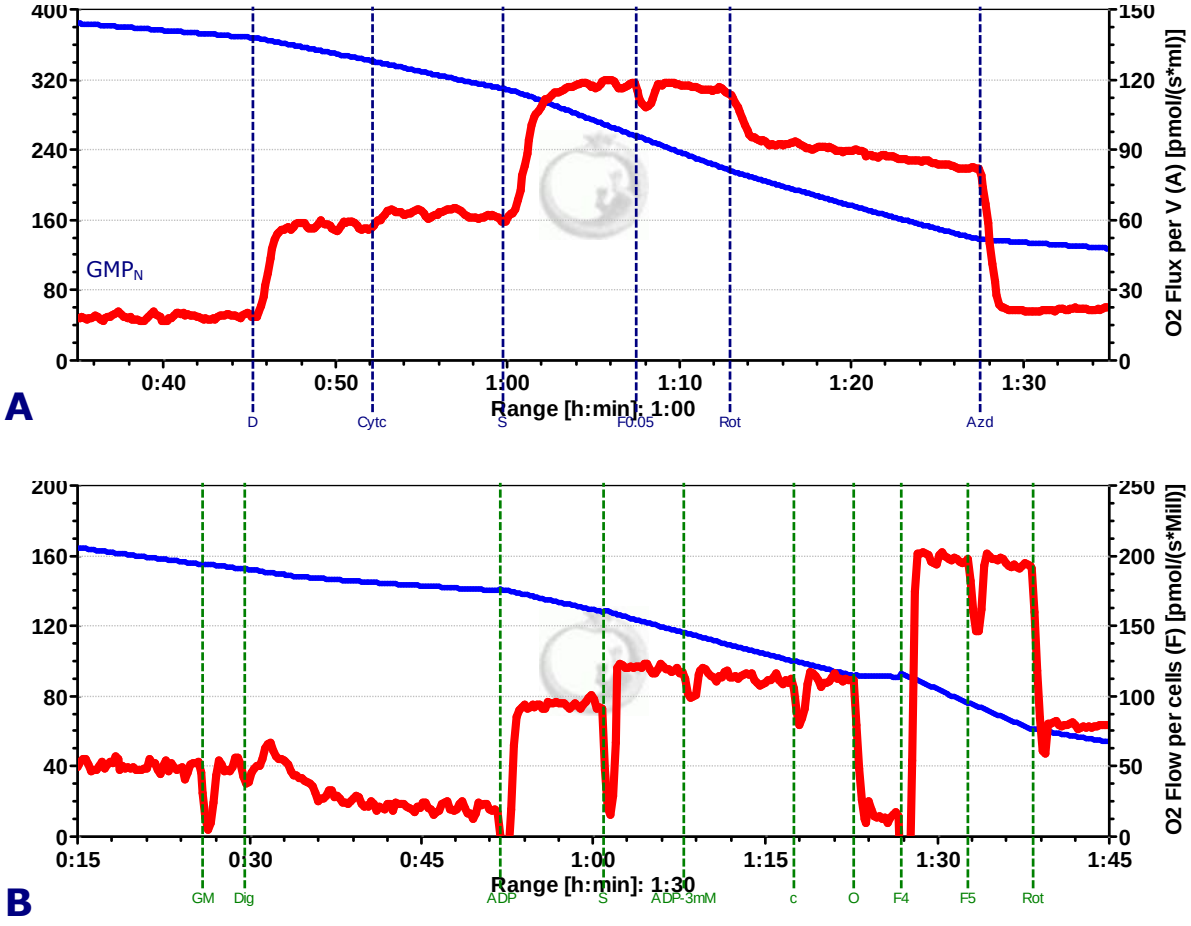


Figure 2. Multiple substrate-inhibitor protocols for high-resolution respirometry in permeabilized muscle fibres and permeabilized cells. Measurements at 37 °C in the 2 ml chamber of the OROBOROS Oxygraph-2k in mitochondrial respiration medium (MiR05). Blue lines: O_2 concentration [µM]; red lines: O_2 flux or flow. **A**: Permeabilized mouse skeletal muscle fibres: PGM_N+D+*c*+S+F+Rot+Azd. PGM pyruvate+glutamate+malate, c cytochrome *c*, S succinate, F FCCP, Rot rotenone, Azd azide. Succinate stimulates flux two-fold, and the phosphorylation system is not limiting (no stimulation by uncoupling with F) (IOC39). **B**: Fibroblasts (NIH3T3; $0.24 \cdot 10^6$ cells/ml): C_e+GM+Dig:GM_N+D+S+*c*+Omy+F+Rot+Ama. After measurement of routine endogenous respiration, glutamate+malate (GM) were added, and cells were permeabilized by digitonin (Dig), inducing state GM_N (LEAK; no adenylates added). ADP (1 mM) stimulated respiration inducing state GM_P (OXPHOS). Succinate (S) increased respiration with convergent Complex I+II electron input (GMS_P). 3 mM ADP and 10 µM cytochrome *c* were without stimulatory effect ($GMSc_P$). After inhibition by oligomycin (LEAK state $GMSc_L$), stimulation by FCCP (ETS state $GMSc_E$) indicated strong limitation by the phosphorylation system. Inhibition by rotenone (Rot) revealed a low capacity of respiration on succinate alone, ETS state $Sc(Rot)_E$. Stimulation of coupled flux by succinate is low since the phosphorylation system exerts significant control over pathway flux indicating a high apparent ETS excess capacity.

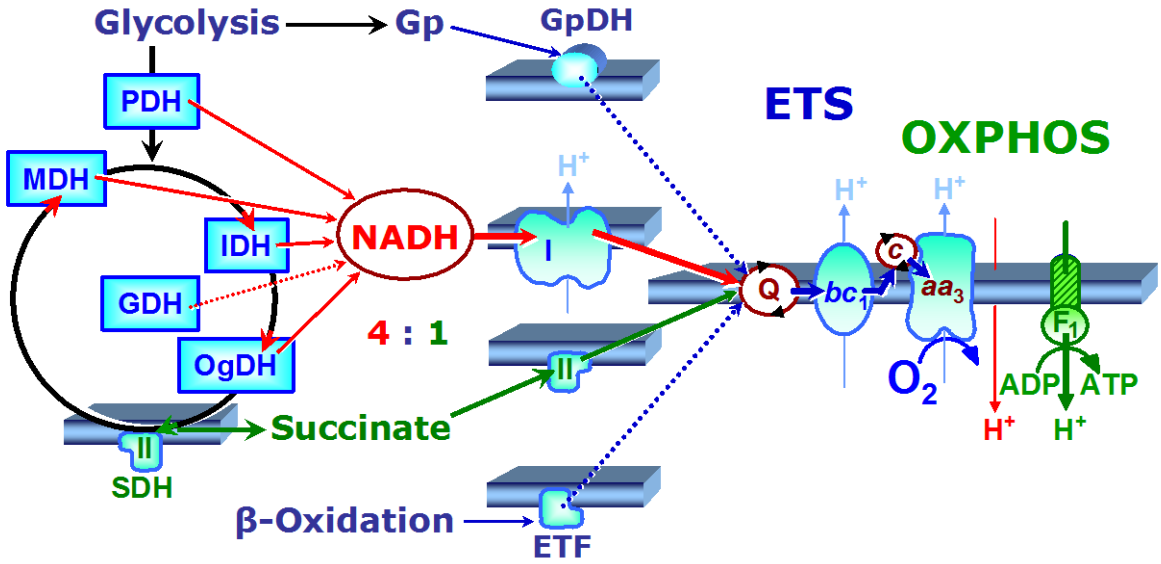
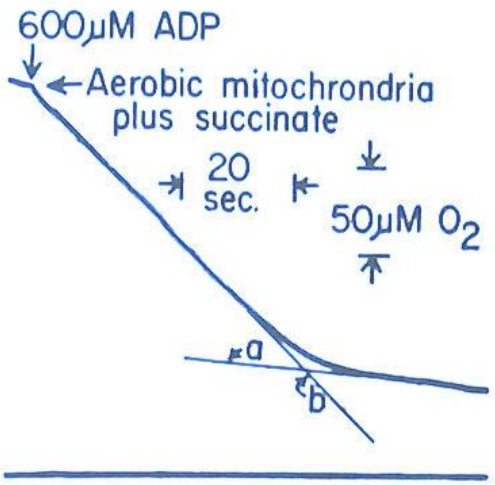


Figure 3. Convergent electron transfer at the levels of the Q-junction (Complexes I+II, GpDH and ETF) and at the level of the NADH pool. Dehydrogenases are PDH, pyruvate DH; MDH, malate DH; IDH, isocitrate DH; OgDH, oxoglutarate DH, SDH, succinate DH - yielding a NADH:succinate ratio of 4:1 in the full TCA cycle. GDH, glutamate DH.

2. Historical Perspectives of Protocols with Substrate Combinations: Beyond the State 3 or ETC Paradigm of Bioenergetics

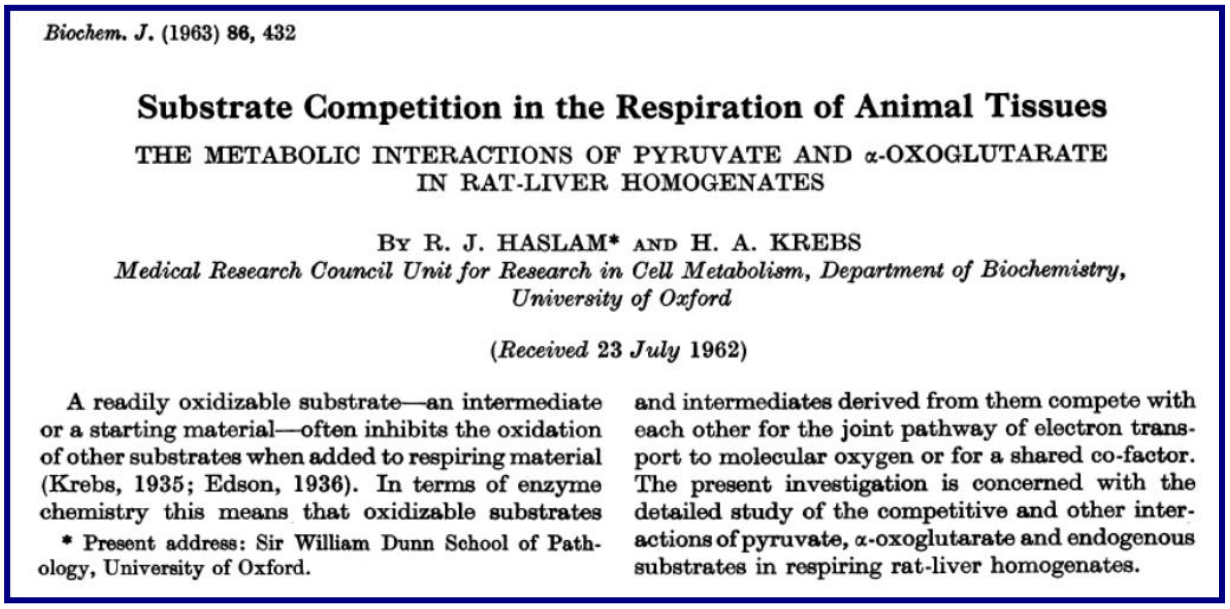
Convergent electron flow through Complexes I+II (Hatefi et al 1962) is not warranted when analyzing site-specific H^+:e and ADP:O ratios. Then segments of the electron transfer system are separated into linear thermodynamic cascades, forming distinct electron transfer *chains*, using either NADH-linked substrates or the classical succinate+rotenone combination (Chance and Williams 1955; Estabrook 1967) [MiPNet11.04]; [MiPNet11.09]. This analytical approach is now applied mainly in the functional diagnosis of OXPHOS. The experimental separation of convergent electron transfer is common to the extent of establishing an '*ETC paradigm of bioenergetics*' in mitochondrial studies (Gnaiger 2009).



Chance and Williams (1955) Fig. 5A. Recording of the kinetics of initiation and cessation of rapid respiration in rat liver mitochondria.

2.1. Intersubstrate competitions

Restriction of permeability of the inner mitochondrial membrane for various substrates of the TCA cycle raised the question on inter-substrate competition for transport, intramitochondrial concentrations and oxidation (Haslam and Krebs 1963).



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Substrate Competition in the Respiration of Animal Tissues

THE METABOLIC INTERACTIONS OF PYRUVATE AND α-OXOGLUTARATE IN RAT-LIVER HOMOGENATES

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A readily oxidizable substrate—an intermediate or a starting material—often inhibits the oxidation of other substrates when added to respiring material (Krebs, 1935; Edson, 1936). In terms of enzyme chemistry this means that oxidizable substrates and intermediates derived from them compete with each other for the joint pathway of electron transport to molecular oxygen or for a shared co-factor. The present investigation is concerned with the detailed study of the competitive and other interactions of pyruvate, α-oxoglutarate and endogenous substrates in respiring rat-liver homogenates.

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Succinate accumulation is decreased by malate, and titration of phosphate or malate in the presence of succinate+rotenone progressively inhibit respiration of rat liver mitochondria (Harris and Manger 1968). In general, '*when two substrates are presented together the respiratory rate obtainable with maximal stimulation by uncoupler can exceed, be equal to or be less than the sum of the rates obtained with the respective substrates separately*' (Harris and Manger 1969). Various mechanisms for explaining these different effects have been recognized, in particular that '*the stimulation of the oxidation of either malate or succinate by the addition of glutamte is due to the removal of oxaloacetate by transamination'* (Harris and Manger 1969; see figure in Section 6). When β-hydroxybutyrate is added to NADH-linked substrates (either citrate, malate, glutamate, oxoglutarate or pyruvate), then '*with these pairs the total rate of oxidation obtainable is equal to the sum of the respective rates measured separately*'. Since the '*respiratory rate obtainable from other pairs of substrates can be less than the sum of the separate rates, even though there is no known inhibition of the enzymes by the conjugate substances*', the available conclusion was that '*in this circumstance mutual competition between the two anions for permeation and accumulation is presumed*' (Harris and Manger 1969).

It is important to note that these investigations were based on non-coupled flux, removing any downstream limitation of flux by the phosphorylation system. Without the concepts of (i) a shift of flux control under different metabolic conditions of substrate supply to different enzyme-catalyzed steps, and (ii) convergent versus linear pathways, however, interpretation of these results on rat liver mitochondria was only possible in terms of intersubstrate competition of substrate transport. The

focus, therefore, was on inhibitory mechanisms. Although stimulation of respiration of rat liver mitochondria was observed when succinate was added to pyruvate (without succinate+rotenone as a control for an additive effect), the interpretation was that '*succinate oxidation did not inhibit pyruvate oxidation*' (König et al 1969). Although these early studies clearly showed that succinate plays an important regulatory role for mitochondrial respiration in the presence of NADH-related substrates, they appear to have had little or no influence on later concepts on mitochondrial respiratory capacity and respiratory control.

2.2. Scattered observations with substrate combinations

It is difficult to trace the history of observations on mitochondrial respiration with specific substrate combinations that lead to convergent electron flow through Complexes I+II (Hatefi et al 1962) or through Complex I and glycerophosphate dehydrogenase. The reason for this difficulty is related to the apparent lack of an explicit conceptual framework, rendering valuable results scattered as observations without specific interpretation. Occasionally, glutamate+succinate has been used to support respiration, without comparison of flux with different substrates (Chance 1965; Sugano et al 1974; Wilson et al 1988), or observing a 2.5-fold increased respiration in the LEAK state with GMS than with GM (Rumsey et al 1990). Results can be found occasionally on ADP-activated OXPHOS capacity (*P*) with Complex I+II substrate combinations.

(i) The group of Alberto Boveris reports respiration with glutamate+succinate (GS_P) which is 1.9-fold higher than glutamate+malate (GM_P) in rat heart mitochondria (Costa et al 1988). While no values were reported for succinate+rotenone in heart, liver mitochondria were studied in the same publication with succinate+rotenone, $S(Rot)_P$, and GM_P without comment, yet rat liver respiration was measured in states GS_P and GM_P in a later work by the same group (González-Flecha et al 1993), and again in a comparison of liver and muscle (Llesuy et al 1994). The GM_P/GS_P flux ratios were 0.8 and 0.7 in liver (male Wistar and female Sprague-Dawley, respectively). No conclusion on an additive effect of the Complex I+II substrates is possible, since succinate alone (+rotenone) supports a higher flux than glutamate+malate in liver mitochondria and permeabilized liver tissue (Kuznetsov et al 2002). In fact, the $GM_P/S(Rot)_P$ flux ratio is 0.6 (Costa et al 1988). The GM_P/GS_P flux ratios of 0.5 and 0.8 for rat heart and muscle mitochondria (Costa et al 1988; Llesuy et al 1994) cannot be interpreted without direct comparison to flux in state $S(Rot)_P$.

(ii) Jackman and Willis (1996) report an additive effect of multiple substrates on mitochondrial respiration: The sum of OXPHOS activities with glycerophosphate (Gp_P) and pyruvate+malate (PM_P) added up to the respiration measured in state $PMGp_P$. The physiological importance of this additive effect of convergent electron flow can be evaluated only with information on the additive succinate effect, since subsequent addition of Gp may then exert a lower stimulatory effect on respiration from state PMS_P to $PMSGp_P$.

(iii) Kuznetsov et al (1996) note in the methods section, that respiration of permeabilized cardiac fibres was measured in a medium containing "10 mM glutamate + 5 mM malate as mitochondrial substrates or additionally 10 mM succinate and 0.08 mM cytochrome *c*". Surprisingly, the fundamentally different effects of succinate and cytochrome *c* are neither distinguished nor even presented. Later, Kunz et al (2000) report a GM_P/GMS_P ratio of 0.7 in permeabilized human muscle fibres. The apparent excess capacity of cytochrome *c* oxidase is lower with reference to the higher flux in state GMS_P, compared to the lower GM_P reference flux. This finding was interpreted by Kunz et al (2000) as a salient feature of permeabilized fibres (or intact cells) in contrast to isolated mitochondria, rather than the consequence of the multiple substrate supply. A less misguided interpretation might have been chosen with reference to the data on mitochondria isolated from rat muscle (Llesuy et al 1994), and to the GM_P/GS_P ratio of 0.5 and 0.7 for isolated mitochondria from pigeon skeletal muscle and human vastus lateralis (Rasmussen and Rasmussen 1997; 2000).

2.3. Maximum flux: Functional assays and non-coupled cells

(iv) Hans and Ulla Rasmussen developed the concept of 'functional assays of particular enzymes' by using various substrates and substrate combinations in respiratory studies of mitochondria isolated from skeletal muscle (Rasmussen and Rasmussen 1997; 2000). A functional assay is based on the stimulation of flux to a maximum, which then is limited by a defined system or particular enzyme. Their concept on the application of multiple substrates (glutamate+succinate) has been largely ignored in the literature, perhaps on the basis of the argument that flux control is distributed over several enzymes along a pathway. Limitation by a single enzyme, which then has a flux control coefficient of 1.0, is a rare event (Rossignol et al 2003), even under conditions of a 'functional assay'. Importantly, however, the 1.4- or even 2.0-fold higher flux in state GS_P (Rasmussen and Rasmussen 1997; 2000) compared to the conventional State 3 paradigm (states PM_P, GM_P and $S(Rot)_P$) raises the critical issue of the physiologically appropriate reference state for measuring flux control coefficients and excess capacities of a particular enzyme such as cytochrome *c* oxidase (Garedew et al 2006). For general consideration, Rasmussen et al (2001) state: "The tricarboxylic acid cycle cannot be established in optimal, cyclic operation with isolated mitochondria, but parts of the *in vivo* reaction scheme may be realized in experiments with substrate combinations".

(v) Attardi and colleagues dismiss isolated mitochondria as a suitable model for respiratory studies, on the basis of the fact that 'State 3' in permeabilized cells (using Complex I-linked substrates only) is low compared to endogenous respiration of intact cells, which then "raises the critical issue of how accurately the data obtained with isolated mitochondria reflect the *in vivo* situation" (Villani and Attardi, 1997). This ignores the scattered observations on multiple substrate effects (see also Kunz et al 2000). Low OXPHOS capacity may be directly related to

artefacts in the isolation of mitochondria (Rasmussen and Rasmussen 1997; Gnaiger et al 2000b). Thus the 'ETC paradigm of State 3' is converted into a paradigm of maximum flux, obtained by studying 'intact' cells in the non-coupled state. Whereas it is highly informative to uncouple intact cells and thus observe maximum respiration through the ETS (Steinlechner-Maran et al 1996; Renner et al 2003; Hütter et al 2004; Gnaiger 2008), cells are not intact after uncoupling. This trivial fact is dismissed under the paradigm of maximization of flux (italics): "KCN titration assays, carried out on *intact uncoupled cells*, have clearly shown that the COX capacity is in low excess (16-40%) with respect to that required to support the endogenous respiration rate" (Villani et al 1998). Uncoupling eliminates any flux control by the phosphorylation system, hence the reference state of the non-coupled cell is nonphysiological, unless the phosphorylation system exerts no control over coupled respiration.

2.4. Mitochondrial Physiology and Mitochondrial Pathways

A rapidly growing number of studies on various tissues and cells points to the importance of the additive effect of substrate combinations on OXPHOS capacity (reviewd by Gnaiger 2009) [MiPNet12.13]. Convergent CI+II electron flow into the Q-junction resolves discrepancies between intact cells and mitochondria. This additive effect indicates a high down-stream excess capacity of respiratory Complexes including cytochrome *c* oxidase (CIV) over Complexes I and II. Convergent electron transfer yields a maximum (additive) effect on OXPHOS when cytochrome *c* oxidase and the phosphorylation system exert a minimum (zero) flux control. Convergent electron transfer corresponds to the operation of the TCA cycle and mitochondrial substrate supply *in vivo*. In isolated mitochondria and permeabilized cells or tissue, conventional measurements of State 3 with pyruvate+malate, glutamate+malate or succinate+rotenone underestimate OXPHOS capacity, since external succinate is required for securing full operation of the TCA cycle and convergent CI+II electron flow under these conditions. The concept of 'convergent electron transfer at the Q-junction' challenges the conventional terminology of a mitochondrial 'electron transfer chain'. Specifically designed multiple substrate-uncoupler-inhibitor titrations (SUIT) extend experimental protocols for the diagnosis of mitochondrial respiratory function. By establishing the reference state of maximum *coupled* respiration, convergent CI+II electron flow provides the proper basis for (i) quantifying excess capacities and interpreting flux control by various enzymes such as cytochrome *c* oxidase and components such as the phosphorylation system, and (ii) evaluation of specific enzymatic defects in mitochondrial respiratory physiology and pathology.

The ratio of OXPHOS capacity (*P*; coupled, State 3) to the capacity of the electron transfer system (*E*; non-coupled) is the *P/E* ratio which yields important information on the limitation of respiration by the phosphorylation system.

3. Pyruvate+Malate+Glutamate: PMG

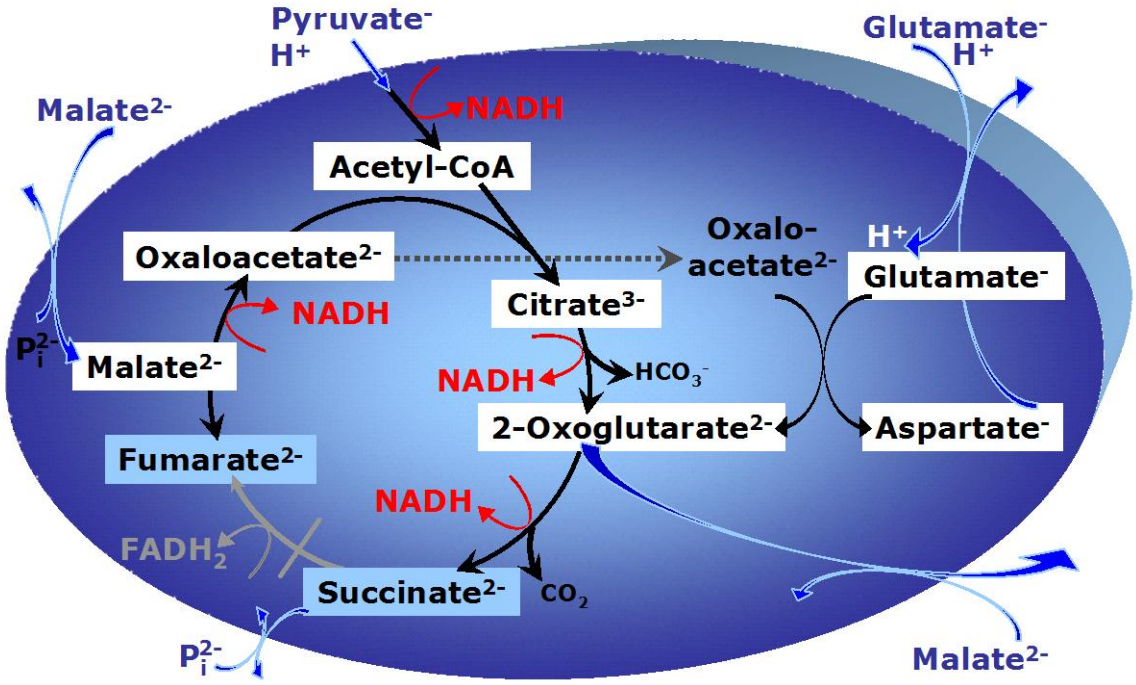

Figure 4. Respiratory capacity through Complex I may be limited by substrate supply. The addition of pyruvate and glutamate with malate (PMG; compare Fig. 3) yields an estimation of respiration in the presence of both pyruvate and the malate-aspartate shuttle (Digerness and Reddy 1976).

Mitochondria from red muscle fibres (rabbit soleus) exhibit a 15% higher flux in state PMG$_P$ (Fig. 4) compared to PM$_P$, whereas white muscle fibre mitochondria (rabbit gracilis) show a slight inhibition by glutamate added to PM$_P$ (Jackman and Willis 1996). Paradoxically, a significant inhibition of flux by addition of pyruvate to GM$_P$ was observed in horse skeletal muscle fibres (cooperation with Dominique Votion, Hélène Lemieux, unpubl.). Addition of glutamate to pyruvate+malate increases respiratory capacity in human skeletal muscle (Winkler-Stuck et al 2005; although PM$_P$ is 16% higher than GM$_P$). There is a strong additive effect of the PMG-substrate combination on respiratory capacity of mouse heart fibres (Lemieux et al 2006).

4. Pyruvate+Malate+Succinate: PMS

Full operation of the TCA cycle in isolated mitochondria or permeabilized tissues and cells requires addition of succinate to the conventional substrates for Complex I (Fig. 5). The TCA cycle is functionally not 'closed' when using the substrate combination pyruvate+malate, when citrate and 2-oxoglutarate are exchanged rapidly for malate by the tricarboxylate and 2-oxoglutarate carrier [MiPNet11.04]. Then succinate dehydrogenase activity is fully dependent on a high external succinate concentration.

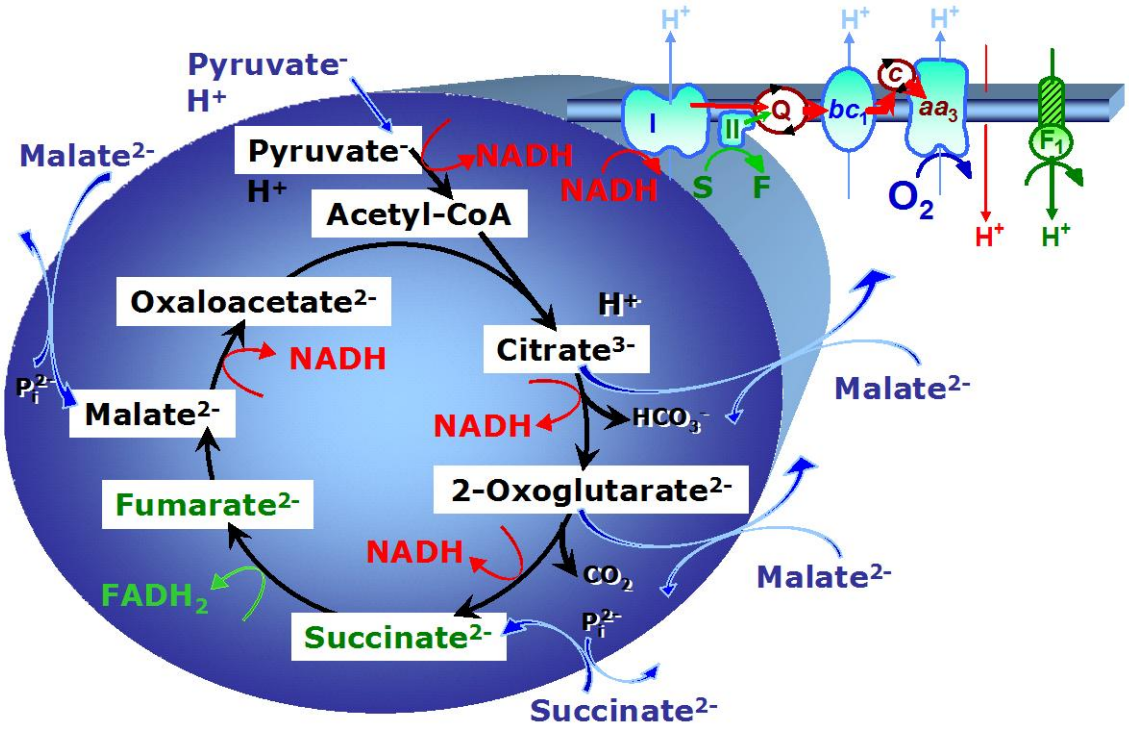

Figure 5. Convergent electron flow to the Q-junction based on incubation with substrate combinations, pyruvate+malate+succinate (PMS).

5. Glutamate+Malate+Succinate: GMS

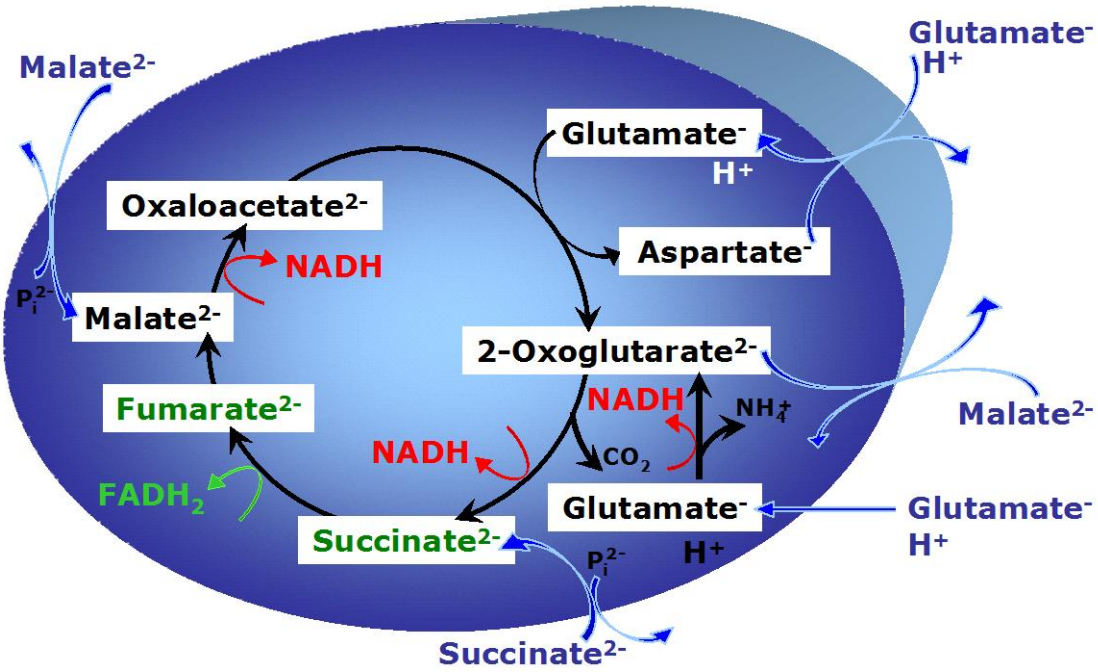

Figure 6. Convergent electron flow to the Q-junction based on incubation with substrate combinations, glutamate+malate+succinate (GMS).

Transaminase catalyzes the reaction from oxaloacetate to 2-oxoglutarate, which then establishes a cycle without generation of citrate. Rasmussen and Rasmussen (1997) reported an increased respiratory flux in OXPHOS activity when using glutamate+succinate (CI+II) compared to glutamate+malate (CI) or succinate+rotenone (CII). This suggests an additive effect of convergent electron input into Complexes I+II. In human skeletal muscle mitochondria (25 °C), Rasmussen and Rasmussen (2000) obtained CI/CI+II flux ratios of 0.7 (0.6) for OXPHOS (or ETS) with glutamate+malate (8+4 mM) and glutamate+succinate (4+8 mM), and CII/CI+II flux ratios of 0.8 (0.6) for OXPHOS (or ETS). Since glutamate alone supported only 49% of OXPHOS flux with glutamate+malate, addition of malate to their glutamate+succinate assay would have been of interest. Identical GM_P/GS_P or GM_P/GMS_P ratios of 0.7, however, were reported for isolated mitochondria (Rasmussen and Rasmussen 2000; Capel et al 2005) and permeabilized fibres (Kunz et al 2000). The GM_P/GMS_P ratio in skeletal muscle of rats fed on various diets ranges from 0.7 to 0.8 (Garait et al 2005). Due to a lower H^+/O_2 stoichiometry in succinate ($FADH_2$) respiration compared to NADH-related respiration (two versus three coupling sites), the CI/CI+II ratio is much lower for LEAK respiration (State 4; 0.3 to 0.4; Garait et al 2005).

In human skeletal muscle, the phosphorylation system is more limiting at the highest OXPHOS activity with glutamate+succinate, at a *P/E* ratio (GS_P/GS_E) of 0.69 versus 0.80 with glutamate+malate (Rasmussen and Rasmussen 2000; see also Boushel et al 2007). Failure of obtaining a further stimulation of coupled OXPHOS in human skeletal muscle mitochondria with GMS by uncoupling (Kunz et al 2000) may be explained by the high FCCP concentration applied (10 µM) which is known to inhibit respiration (Steinlechner-Maran et al 1996). In mouse skeletal muscle, however, the *P/E* ratio is actually 1.0 (Aragones et al 2008), which contrasts with the significant limitation of OXPHOS capacity by the phosphorylation system in humans (Gnaiger 2009).

6. Pyruvate+Malate+Glutamate+Succinate: PMGS

2-oxoglutarate is produced through the citric acid cycle from citrate by isocitrate dehydrogenase, from oxaloacetate and glutamate by the transaminase, and from glutamate by the glutamate dehydrogenase. If the 2-oxoglutarate carrier does not outcompete these sources of 2-oxoglutarate, then the TCA cycle operates in full circle with external pyruvate+glutamate+malate+succinate (Fig. 7).

7. Additive Effect of Glycerophosphate Dehydrogenase and Electron-Transferring Flavoprotein

On the outer face of the inner mitochondrial membrane, mitochondrial glycerophosphate dehydrogenase oxidizes glycerophosphate to dihydroxyacetone phosphate, reducing a flavin prosthesic group that donates its reducing equivalents to the electron transfer system at the

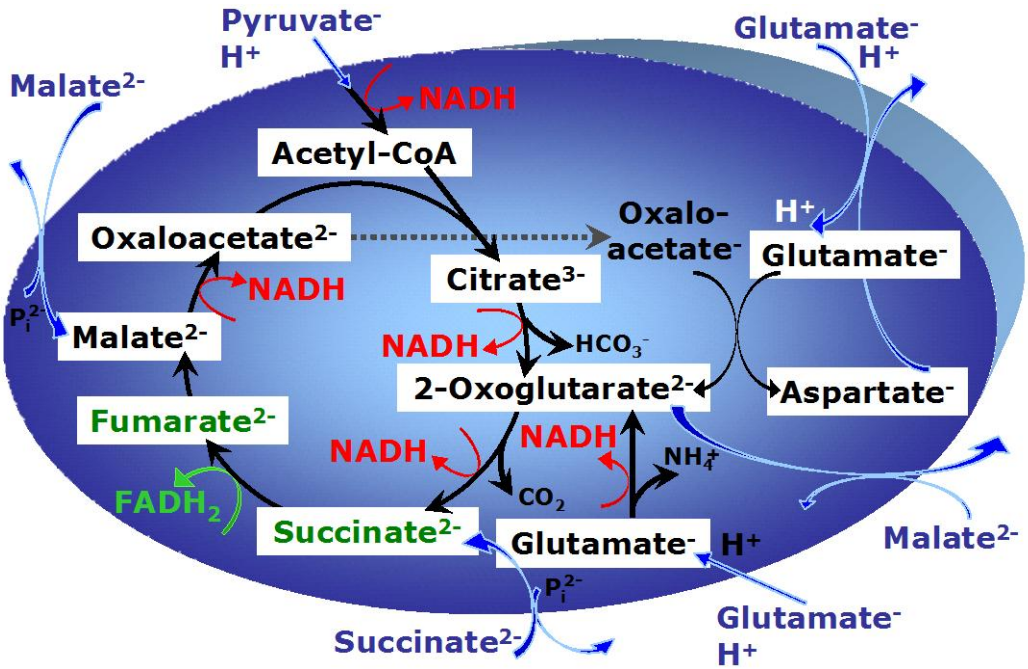

Figure 7. Convergent electron flow to the Q-junction based on incubation with substrate combinations, pyruvate+malate+ glutamate+succinate (PMGS).

level of CoQ. Electron-transferring flavoprotein (ETF) is located on the matrix face of the inner mitochondrial membrane, and supplies electrons from fatty acid *β*-oxidation to CoQ.

Glycerophosphate oxidation is 10-fold greater in rabbit gracilis mitochondria (fast-twitch white muscle; 99% type IIb) compared to soleus (slow-twitch red muscle; 98% type I). Both types of skeletal muscle mitochondria exhibit additive pyruvate and glycerophosphate oxidase activities (Jackman and Willis 1996).

Oxygen flux in the presence of glycerophosphate is increased by subsequent addition of succinate to brown adipose tissue mitochondria (Rauchova et al 2003). It has yet to be shown if there is an additive effect of applying these two substrates, or if succinate+rotenone respiratory capacity is already sufficient for supporting the maximum flux.

Red and white rabbit muscle mitochondrial respiration is slightly increased when palmitoylcarnitine is added to PM_P (Jackman and Willlis 1996). Similarly, ATP production in mitochondria isolated from human muscle is higher with a substrate combination (malate+pyruvate+2-oxoglutarate+palmitoylcarnitine) that supports convergent electron input into the Q-junction through Complex I and ETF, than with electron input into either Complex I or II (Tonkonogi et al 1999; Short et al 2005). Human skeletal muscle mitochondria and permeabilized fibres respire at 0.60 of GM_P with malate+palmitoylcarnitine (vastus lateralis; Rasmussen and Rasmussen 2000) or malate+octanoylcarnitine (vastus lateralis; Gnaiger et al 2005). This ratio (relative to PM_P) was also reported for mitochondria isolated from rat extensor digitorum longus muscle (mainly type II fibre type), but is 0.95 in rat soleus muscle (type I fibre type; Mogensen and Sahlin 2005).

8. Implications

Application of physiological substrate combinations is a hallmark of the extension of mitochondrial bioenergetics to mitochondrial physiology. Conventional studies with various substrates for NADH-related dehydrogenases prevent the simultaneous activation of Complex II or other convergent pathways into the Q-junction (glycerophosphate dehydrogenase, ETF). Convergent electron input into the Q-cycle characterizes mitochondrial function *in vivo*, with a minimum of 1/5th to 1/4th of electron flow through Complex II even for respiration on pure carbohydrate.

(a) For appreciation of the diversity of mitochondrial function in different animals and tissues, mitochondrial respiratory capacity has to be generally re-assessed with application of substrate combinations appropriate for a complete operation of the citric acid cycle.
(b) Interpretation of excess capacities of various components of the electron transfer system and of flux control coefficients is largely dependent on the metabolic reference state. Appreciation of the concept of the Q-junction will provide new insights into the functional design of the respiratory system.
(c) The conventional view of a drop of mitochondrial membrane potential with an increase of flux from the LEAK to OXPHOS state (State 4 to State 3) will have to be modified, based on the appreciation of the important control of flux by substrate supply. The relation between membrane potential and flux is reversed when an increase in flux is affected by a change in substrate supply.
(d) Based on the relationship between ROS production and reversed electron flow from Complex II to Complex I, multiple substrates have been supplied in investigations of oxidative stress related to mitochondrial metabolism (Hansford et al 1997; Capel et al 2005; Garait et al 2005; Zoccarato et al 2007; Muller et al 2008). The dependence of ROS production on membrane potential and metabolic state will have to be investigated further, to resolve pertinent controversies on the role of mitochondria in cellular ROS production.

A functional association of Complex I and Complex III, but not of Complex II and Complex III in bovine heart mitochondria (Bianchi et al 2004), suggests that convergent electron input into Complexes I+II may not or only partially proceed through a common CoQ pool, and that the actual junction of electron transfer occurs downstream of Complex III. No functional association was apparent with Complex IV.

References:

http://www.bioblast.at/index.php/Gnaiger_2012_MitoPathways_References