Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements

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Partitioning of ATP generation between glycolysis and oxidative phosphorylation is central to cellular bioenergetics but is cumbersome to measure. We describe here how rates of ATP generation by each pathway can be calculated from simultaneous measurements of extracellular acidification and oxygen consumption. We update theoretical maximum ATP yields by mitochondria and cells catabolizing different substrates. Mitochondrial P/O ratios (mol of ATP generated per mol of [O] consumed) are 2.73 for oxidation of pyruvate plus malate and 1.64 for oxidation of succinate. Complete oxidation of glucose by cells yields up to 33.45 ATP/glucose with a maximum P/O of 2.79. We introduce novel indices to quantify bioenergetic phenotypes. The glycolytic index reports the proportion of ATP production from glycolysis and identifies cells as primarily glycolytic (glycolytic index > 50%) or primarily oxidative. The Warburg effect is a chronic increase in glycolytic index, quantified by the Warburg index. Additional indices quantify the acute flexibility of ATP supply. The Crabtree index and Pasteur index quantify the responses of oxidative and glycolytic ATP production to alterations in glycolysis and oxidative reactions, respectively; the supply flexibility index quantifies overall flexibility of ATP supply; and the bioenergetic capacity quantifies the maximum rate of total ATP production. We illustrate the determination of these indices using C2C12 myoblasts. Measurement of ATP use revealed no significant preference for glycolytic or oxidative ATP by specific ATP consumers. Overall, we demonstrate how extracellular fluxes quantitatively reflect intracellular ATP turnover and cellular bioenergetics. We provide a simple spreadsheet to calculate glycolytic and oxidative ATP production rates from raw extracellular acidification and respiration data.

Cells require energy to run the reactions that maintain their viability, growth, and proper function. The dominant currency of chemical energy in cells is ATP, which is produced mostly by two pathways: glycolysis in the cytosol and oxidative phosphorylation in the mitochondria. The rates of these pathways are cumbersome to measure directly. They can be followed using the fluxes of isotopic tracers, such as 13C and 31P, between metabolic pools (e.g. Refs. 1 and 2). However, they can also be estimated relatively easily and quickly from the rates of linked reactions: extracellular acidification resulting from glycolytic conversion of uncharged glucose to 2 lactate− plus 2 H+ and oxygen consumption to oxidize pyruvate and other substrates and to support oxidative phosphorylation.

The rate of glycolysis in cell culture has been measured in a variety of ways, including using the rates of production of lactate and protons. Our focus here is on measurement of extracellular proton production, which can quantitatively report the rate of glycolysis to lactate and can be measured using pH electrodes or by fluorescent indicators simultaneously with oxygen consumption in commercial instruments (3–5).

Historically, the rate of oxygen consumption has been measured using Clark oxygen electrodes. These can consume significant amounts of oxygen and therefore work best in well-mixed, bulk aqueous solutions, limiting amenable experimental systems to suspended material, including cells and isolated mitochondria. More recent methods use fluorescent sensors, which have high sensitivity and bind negligible amounts of oxygen and can therefore be used to measure oxygen consumption rates in small volumes of aqueous media above adherent mitochondria and cell cultures. Modern commercial fluorescence-based instruments for the measurement of oxygen consumption rates (3) incorporate sophisticated corrections for oxygen diffusion (6) and have been described extensively in both practical (4, 7) and theoretical contexts (8).

Combining the extracellular measurement of rates of acidification and oxygen consumption into one assay provides a powerful way to assess the total energy metabolism of a cell (i.e. the total rate of ATP cycling through production by both glycolysis and oxidative reactions and consumption by the pathways that impose an ATP demand). A description of the bioenergetic phenotype of a cell is the highly desired outcome of many investigations and can be an important reporter of cellular status and behavior. However, despite the potential utility of simultaneous measurement of glycolysis (by extracellular acidification) and oxidative phosphorylation (by oxygen consumption) to quantify ATP turnover, most current analyses directly
compare extracellular acidification rate (ECAR\(^2\)) in mpH units/min to oxygen consumption rate (OCR, in pmol O\(_2\)/min). This direct comparison can be very misleading, for five main reasons.

First, the rate of change of pH depends on the buffering power (BP) of the medium (BP = change in pH/nmol of H\(^+\)). A given rate of glycolytic proton production will cause high rates of pH change in a lightly buffered medium but low rates of pH change in a well-buffered medium. Correction for the buffering power of the medium and conversion of ECAR to total proton production rate (PPR\(_{tot} = \text{ECAR}/\text{BP}\)) (5) is essential for quantitative interpretation of raw data.

Second, during catabolism of glucose, there are two main pathways that contribute to extracellular acidification: conversion of glucose to lactate (PPR\(_{glyc}\)) and conversion of glucose to bicarbonate (PPR\(_{bicarb}\)). Acidification associated with bicarbonate production can be small, intermediate, or dominant, or all of these within one experiment, so it must be assessed and subtracted before the proton production rate can be equated to the lactate production rate (PPR\(_{glyc} = \text{PPR}_{tot} - \text{PPR}_{bicarb}\)) (5).

Third, pyruvate produced by glycolysis has two different fates: reduction to lactate or oxidation to bicarbonate. For correct assessment of the rate of glycolysis to pyruvate, with either subsequent fate of the pyruvate, the rates of these two components must be converted to the same units (e.g. glucose consumed/min) and summed.

Fourth, even when the rates of glycolysis and oxidative metabolism are correctly assessed, the two pathways produce very different amounts of ATP per glucose. To characterize a cell as “very glycolytic,” the majority of its ATP should come from glycolysis rather than oxidative reactions. The rates of the two pathways should therefore be converted into the same units (ATP production) before they are compared. As shown in Fig. 1, glucose catabolism produces 2 ATP/glucose from glycolysis but up to 31.45 ATP/glucose from oxidative reactions during the complete oxidation of glucose to bicarbonate. This gear

is easily accounted for by comparing the rates of the two pathways in the same units (i.e. ATP produced per time).

Fifth, because of the leak of protons across the mitochondrial inner membrane, not all oxygen consumption is coupled to ATP synthesis, so it is coupled oxygen consumption, not total oxygen consumption, that should be considered.

Once these factors are considered, the rationale for calculating \(J_{\text{ATPglyc}}\) and \(J_{\text{ATPox}}\) is clear. The method we introduce here builds on our previous deconvolution of glycolytic and respiratory sources of acidification (4, 5) and extends this analysis to the calculation of rates of intracellular ATP production by glycolysis and oxidative reactions from extracellular measurements of rates of acidification and oxygen consumption. Once the rates of ATP production are known, we show how they can be used to quantify and interpret classical qualitative indicators of cellular energy metabolism, the Warburg, Crabtree, and Pasteur effects, and to quantify the flexibility of substrate use and the bioenergetic capacity of cells. We use this method to characterize the bioenergetic phenotype of C2C12 myoblasts under different conditions and to assess whether different ATP-consumption reactions draw preferentially on glycolytic or oxidative ATP production.

**Results**

*Updated consensus view of maximum ATP yields by mitochondria and cells catabolizing different substrates*

The yield of ATP from oxidative phosphorylation per oxygen atom ([O]) consumed, the P/O ratio, has been investigated for 70 years using isolated mitochondria. When the mechanism was thought to be analogous to substrate-level phosphorylation, the P/O ratio was assumed to be a small integer, with a maximum value of 3 for oxidation of NAD-linked substrates. Once a chemiosmotic mechanism was accepted, it became clear that the maximum P/O ratio for reducing equivalents entering the electron transport chain is the number of protons pumped out of the matrix (H\(^+/\)O) divided by the number needed to make each ATP as protons re-enter the matrix (H\(^+/\)ATP). H\(^+/\)O and H\(^+/\)ATP are themselves composed of simple combinations of a few small integers resulting from the underlying molecular mechanisms, so the maximum P/O ratio is generally not an integer. Brand (9) summarized the then-consensus values of H\(^+/\)O, H\(^+/\)ATP, and P/O for different substrates, based on empirical measurements refined by theoretical models. The H\(^+/\)O ratio for oxidation of simple NAD-linked substrates by mitochondria was (and remains) 10. The H\(^+/\)ATP ratio at the F\(_{1}\)F\(_{0}\)-ATP synthase is the number of protons driven through the c-ring of the F\(_{0}\) subunit during a complete rotation of the F\(_{1}\) subunit, divided by the 3 ATP synthesized per rotation. Based on the reported 10 c-subunits/c-ring in yeast F\(_{0}\), the value for H\(^+/\)ATP was therefore 10 H\(^+\) transferred/rotation, plus 3 H\(^+\) used to translocate the 3 ATP made or consumed, giving an H\(^+/\)ATP ratio of 13.3. Combining the H\(^+/\)O and H\(^+/\)ATP values gave 10 × 3/13 for the maximum P/O ratio for oxidation of simple NAD-linked substrates by mitochondria, close to 2.308.

In Fig. 1A, we update the H\(^+/\)ATP and P/O values in Ref. 9, based on subsequent refinement of the structural model of F\(_{0}\).
There are now thought to be eight c-subunits in the c-ring of the mammalian F₁Fₒ-ATP synthase, not 10 (10). Assuming 8 H⁺ translocated per rotation, H⁺/ATP changes from 13.3 to 11.3, altering the theoretical P/O ratios and potential yields of ATP. For example, for oxidation of pyruvate plus malate by isolated mitochondria, the revised maximum P/O ratio is 10 × 3/11, close to 2.727 (Fig. 1A) and substantially larger than the previous value of 2.308 (9).

Fig. 2 shows the pathways involved when glucose (or glycogen) is used as the substrate for cellular ATP production, and Fig. 1A provides the associated accounting. During cellular glycolysis to lactate, the net yield is 2 ATP/glucose (or 2.9 ATP/glucose unit in glycogen, assuming 90% α-1,4 glycosidic bonds). During complete oxidation of glucose, glycolysis yields 2 ATP/glucose, and oxidative phosphorylation plus the tricarboxylic acid cycle yields up to 31.45 ATP/glucose; the maximum total yield is 33.45 ATP/glucose and the maximum overall P/O ratio is 2.78. During complete oxidation of glycogen, these values are 2.9, 31.45, 34.35, and 2.86, respectively. The full set of updated values for the oxidation of a variety of substrates by mitochon-

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### A

| Substrate and pathway | Reaction | Stoichiometries | Maximum ATP from glycolysis | Maximum ATP from oxidative metabolism | Maximum yields per substrate and O
|-----------------------|----------|----------------|-----------------------------|---------------------------------------|---------------------------------------------|
|                       |          |                | (mol ATP)                   |                                       |                                             |
| Glucose to lactate    | C₆H₁₂O₆ → 2C₃H₅O₇ + 2H⁺ | 1 0 -2 4 2 | 1.45 | 20 | 2 | 92 | 2 | 92 | 104 | 31.45 | 33.45 | 2.78 |
| Glucose to CO₂ (malate-aspartate shuttle) | C₆H₁₂O₆ → 1[12H] + 6HCO₃⁻ | 1 12 -2 4 2 | 2.145 | 20 | 2 | 92 | 110 | 31.45 | 33.45 | 2.78 |
| Glucose to CO₂ (glycerol 3-P shuttle) | C₆H₁₂O₆ → 1[12H] + 6HCO₃⁻ | 1 12 -2 4 2 | 2.145 | 20 | 2 | 92 | 104 | 31.45 | 31.45 | 2.651 |

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### B

| Substrate and pathway | Maximum ATP per mol [O] (P/O) | C
|-----------------------|-------------------------------|---------------------------------------------|
| Glucose to CO₂ (malate-aspartate shuttle) | 0.16 0.12 2.50 2.78 | ![Equation](J_Biol_Chem_2017_292(17)_7189–7207_7191)
| Glucose to CO₂ (glycerol 3-P shuttle) | 0.16 0.12 2.36 2.651 | ![Equation](J_Biol_Chem_2017_292(17)_7189–7207_7191)
| Glycogen (90% α-1,4) to CO₂ (malate-aspartate shuttle) | 0.24 0.12 2.50 2.86 | ![Equation](J_Biol_Chem_2017_292(17)_7189–7207_7191)
| Glycogen (90% α-1,4) to CO₂ (glycerol 3-P shuttle) | 0.24 0.12 2.36 2.73 | ![Equation](J_Biol_Chem_2017_292(17)_7189–7207_7191)
| Pyruvate to CO₂ | 0 0.145 2.509 2.654 | ![Equation](J_Biol_Chem_2017_292(17)_7189–7207_7191)
| Palmitate (16:0) to CO₂ | 0 0.083 2.372 2.455 | ![Equation](J_Biol_Chem_2017_292(17)_7189–7207_7191)
| Oleate (18:1) to CO₂ | 0 0.089 2.385 2.474 | ![Equation](J_Biol_Chem_2017_292(17)_7189–7207_7191)
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dria and cells and the assumptions behind the calculations are detailed in Fig. 1A.

Definition of amounts and rates of glycolytic and oxidative ATP production

The vast majority of ATP made in cells comes either from
glycolysis (ATPglyc) or from oxidative reactions (ATPox); the corresponding rates (denoted by \( f \)) are \( J_{ATPglyc} \) and \( J_{ATPox} \). If we apply the principles of modular kinetic analysis (11), the complex network of metabolic reaction rates and concentrations of metabolic intermediates involved in ATP turnover collapses to a simple model, represented in Fig. 2A, in which \( J_{ATPglyc} \) and \( J_{ATPox} \) are represented by arrows that point to a common intermediate (ATP, ATP/ADP, phosphorylation potential, etc.), describing the two major pathways by which ATP is generated in cells. A third arrow \( (J_{ATP, consumption}) \) points away from this intermediate and represents all of the pathways that consume ATP.

The relevant reactions of ATP production in the steady state are made explicit in Fig. 2B. We define \( \text{ATPglyc} \) as the net amount of ATP made by substrate-level phosphorylation dur-
ing glycolytic conversion of glycogen or glucose (or other sugars) to pyruvate. The pyruvate has two possible reaction fates (Fig. 2B); therefore, the total $J_{ATP, glyc}$ is the sum of these two rates. Pyruvate can be reduced to lactate using the NADH produced at glyceraldehyde 3-phosphate dehydrogenase, driving no further ATP synthesis; the amount of ATP produced during glucose catabolism by this route can be calculated from the rate of lactate production. Lactate production can be measured directly or calculated from the associated extracellular acidification (PPRglyc; see below). Alternatively, the pyruvate can be oxidized by the mitochondria, in which case the reducing equivalents on glycolytic NADH are also oxidized by the mitochondria, and the further ATP that is generated from this NADH is counted as part of $J_{ATP, ox}$. This portion of $J_{ATP, glyc}$ can be calculated from the associated rate of oxygen consumption, assuming full oxidation of the pyruvate to bicarbonate (see below). Note that this definition of ATPglyc includes all of the ATP made during glycolysis to pyruvate, not just the ATP that is made with lactate as the final product. This definition of ATPglyc is rational because it describes overall flux through glycolysis. However, it contrasts with a different common definition of glycolytic ATP production, which is strictly that of glycolysis to lactate. It is important to be clear about which definition is being used in the literature in any particular context.

We define $J_{ATP, ox}$ as the net amount of ATP made during oxidative metabolism, both oxidative phosphorylation (using reducing equivalents from both glycolysis and pyruvate oxidized to bicarbonate) and substrate-level phosphorylation at succinyl-CoA synthetase as carbon flows around the tricarbox-
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ylic acid cycle. The reducing equivalents carried on cytosolic NADH derived from glycolysis can enter the mitochondrial matrix by two different routes, the malate-aspartate shuttle and the glycerol 3-phosphate shuttle (Fig. 2, B and C); the two routes give slightly different ATP yields. The rate of oxidative ATP production \(J_{ATPox}\) can be calculated from the rate of oxygen consumption for complete oxidation of a defined substrate (Figs. 1 and 2).

The total rate of ATP production \(J_{ATPproduction} = J_{ATPglyc} + J_{ATPox}\). In the steady state, \(J_{ATPproduction}\) is equal to the total rate of ATP consumption, \(J_{ATPconsumption}\).

The model system; C2C12 cells consuming glycogen or glucose by defined pathways

Indirect estimation of rates of ATP production from extracellular acid and oxygen fluxes as described below is a very powerful approach to understanding cellular bioenergetics, providing important information from simple measurements. In support of this view, the reaction stoichiometries for the complete oxidation of different substrates are unambiguous (Fig. 1), the contribution of glycolytic lactate production to overall extracellular acidification can be readily calculated from measured extracellular changes in pH and oxygen concentration (5), and the maximum P/O values predicted by our current understanding of ATP synthesis (Fig. 1) are constant (although subject to possible future change with further refinement of the mechanistic models of the proton pumps).

Although this approach is unambiguous for defined substrates, physiologically relevant mixes of different substrates lead to greater calculation uncertainty. In addition, when cells are growing, flux through anabolic pathways (such as the pentose phosphate pathway) may be quantitatively important. Under these conditions, the relationships between the extracellular measurements and the ATP production by the two major pathways are poorly defined unless the carbon pathways and fluxes are known or assumed. This is because the respiratory quotient (CO₂ produced/O₂ consumed), which is used to define PPRglyc, depends on the substrate and the extent to which it is fully oxidized (although the respiratory quotients for complete oxidation of conventional substrates range only from about 0.7 to 1.2 (5), so assuming a value of 1.0 is usually adequate for semi-quantitative estimation). Similarly, the overall maximum P/O ratio is the weighted mean of the P/O values of all the oxidative pathways. For the complete oxidation pathways described in Figs. 1 and 2, the range of maximum P/O values is small (the value for glycogen is only 17% greater than the value for palmitate), and the assumption of an average value would have only small effects on the calculations. However, other more unusual, partial, or complicated pathways have more extreme respiratory quotients and P/O ratios.

Fortunately, it is possible to avoid these complications when demonstrating the underlying concepts, by designing experiments that minimize the number of parallel metabolic routes. To keep things relatively straightforward, in the present paper, we consider only the simplest case of glucose or glycogen being converted to lactate or fully oxidized to bicarbonate. ATP yields during the metabolism of other substrates or mixtures of substrates could be analyzed in the same way, but these cases require knowledge or assumptions about the relative fluxes through each metabolic pathway, complicating the analysis.

This approach to predicting cellular ATP production is accurate to the extent that the assumptions behind the calculations fit the experimental system. The initial model that we present here assumes that cells given no exogenous substrate metabolize only endogenous glycogen, and when external glucose is provided, this glucose is the sole substrate. In either case, we assume that glucose units are only converted to lactate or fully oxidized to bicarbonate, that reducing equivalents on extramitochondrial NADH enter the matrix primarily (90%) through the malate-aspartate shuttle, and that there are no anabolic reactions or cell growth.

We have chosen a cell model (C2C12 myoblasts) and experimental design that fit these assumptions within the error of our measurements. C2C12 myoblasts were used in previous work that was foundational to the data we present here (4, 5, 12), and in other work using the Seahorse XF extracellular flux analyzer (7). Glucose uptake by C2C12 myoblasts is largely insulin-independent, occurring primarily through the GLUT1 transporter (13, 14). Under our standard experimental conditions, all of the extracellular acidification and oxygen consumption can be quantitatively assigned to glycolysis or oxidative reactions (5).

Cells were assayed in a minimal salts medium following a short period of starvation, conditions very likely to severely slow or stop cell growth during the measurements. Of course, the conclusions reached below are only applicable to this non-growing state; a more sophisticated analysis would be required for growing cells.

Calculation of ATP production rates \(J_{ATPglyc}\) and \(J_{ATPox}\) from extracellular acidification rate and oxygen consumption rate

As diagrammed in Fig. 1C, \(J_{ATPproduction}\) is the sum of \(J_{ATPglyc}\) and \(J_{ATPox}\). \(J_{ATPglyc}\) is itself the sum of two parts. The first is ATP produced by glycolysis to pyruvate that is subsequently converted to lactate. This is calculated from extracellular acidification rate as PPRglyc × ATP/lactate. Because 2 lactates are produced per glucose or glucose residue, ATP/lactate is half the value for ATP/glucose given in Fig. 1A, column g (i.e. 1.0 for glucose, 1.45 for glycogen). The second is ATP produced by glycolysis to pyruvate that is subsequently converted to bicarbonate. This is calculated from the oxygen consumption rate by multiplying the mitochondrial OCR by the P/O ratio attributable to glycolytic production of pyruvate that is then fully oxidized (Fig. 1B, column s), with a conversion factor of 2 to account for the switch from oxygen atoms ([O]) in P/O to molecules \(O_2\) in OCR. Thus, \(J_{ATPglyc}\) to bicarbonate = OCRmito × 2P/Oglyc. Total \(J_{ATPglyc}\) is therefore PPRglyc × ATP/lactate + OCRmito × 2P/Oglyc (Fig. 1C). As a side note, glycolysis to pyruvate precedes both lactate production and oxidation to bicarbonate; for this reason, it is incorrect to use any glycolytic inhibitor that acts before lactate dehydrogenase, such as 2-deoxyglucose, to attempt to distinguish between glycolysis and respiration; both processes will be prevented by such inhibitors when the main substrate is glycogen or glucose. The approach outlined here avoids the need to inhibit glycolysis.

\(J_{ATPox}\) is calculated from the mitochondrial oxygen consumption rate, the portion of the total oxygen consumption

rate that is sensitive to the mitochondrial electron transport inhibitors rotenone and myxothiazol (OCR_{mito} = OCR_{tot} - OCR_{coli}). OCR_{mito} can be further divided into the phosphorylating or coupled rate, which is sensitive to the ATP synthase inhibitor oligomycin (OCR_{coupled} = OCR_{tot} - OCR_{coli}), and the non-coupled or leak rate, which is the mitochondrial OCR in the absence of oligomycin (OCR_{leak} = OCR_{tot} - OCR_{oli}). Comparing OCR before and after oligomycin addition causes a slight underestimate of OCR_{coupled}. The underestimate (<10%) results from the oligomycin-dependent hyperpolarization of the mitochondrial inner membrane, which provokes a slight increase in OCR_{oli} and can be corrected for (15).

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Figure 3. Raw extracellular flux data and its conversion into rates of ATP production (J_{ATPox}). A and B, raw traces of extracellular acidification and oxygen consumption by C2C12 myoblasts. A basal measurement was recorded in the absence of exogenous substrate, followed by the addition of 10 mM glucose, vehicle control (DMSO; maximum concentration < 0.05% (v/v)), 2 μg/ml oligomycin (oli), and 1 μM rotenone with 1 μM myxothiazol (rot/myx). Points within gray regions were assumed to be at or near steady state and were part of the data set used to calculate values shown in sequential columns in C and D. Points are means ± S.E. (error bars) of n = 4 independent experiments. The final protein content of each well was typically 10–15 μg. C, J_{ATP production}, for each time point marked in gray in A and B, divided into J_{ATPglyc} and J_{ATPox}. Aggregate data from multiple experiments, including those in A and B, gave rise to basal n = 24, glucose n = 36, and glucose + oligomycin n = 19. D, data in C further divided into the component reactions of J_{ATPglyc} and J_{ATPox-oxphos}, J_{ATPox-SCS}. J_{ATPox} is divided into ATP production from oxidation of glycolytic NADH (J_{ATPox-glyc}), from oxidation of reducing equivalents generated within the mitochondria (J_{ATPox-oxphos}) and from succinyl-CoA synthetase (J_{ATPox-SCS}).

Experimental determination of rates of ATP production by glycolysis and oxidative metabolism

Here, we apply the calculations above to determine the rates of ATP production by glycolysis, J_{ATPglyc}, and oxidative metabolism, J_{ATPox}, using C2C12 myoblasts in adherent cell culture.

Fig. 3 shows raw extracellular flux data and the overall values and individual components of J_{ATPox} and J_{ATPox} during a partial cell respiratory control assay (8). Fig. 3A shows raw ECAR traces, and Fig. 3B shows raw OCR traces during the course of a single set of experiments. No substrate was present under basal conditions, which were followed by sequential additions at the points indicated of glucose, dimethyl sulfoxide vehicle (DMSO, added as a control for other data sets), oligomycin, and finally rotenone plus myxothiazol. The shaded regions define each condition after the system approached steady state, but before other additions. Under the basal condition, ECAR was low and OCR was high because the cells produced ATP primarily by oxidative phosphorylation from endogenous substrates,
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assumed to be glycogen (see below). After the addition of glucose, ECAR increased, reflecting a shift to glycolysis using the new substrate, and OCR decreased, reflecting the shift away from oxidative phosphorylation (the Crabtree effect). The subsequent addition of oligomycin inhibited the mitochondrial ATP synthase, so ECAR increased, reflecting a greater requirement for glycolytic ATP, and OCR decreased, reflecting the inhibition of oxidative phosphorylation. The remaining mitochondrial OCR represents electron transport driving the cycle of proton pumping and proton leak across the mitochondrial inner membrane. The addition of rotenone plus myxothiazol fully inhibited mitochondrial electron transport, so it had little further effect on ECAR but decreased OCR to the non-mitochondrial rate, which was subtracted from all other OCR values below.

$I_{ATPglyc}$ and $I_{ATPox}$ were calculated as described above (and in the worked example below) from a larger data set that included the data in Fig. 3, A and B (see “Experimental procedures”). Fig. 3C shows the results. We assumed that, under basal conditions, full oxidation of endogenous glycogen drove ATP production. There was no glycolysis to lactate; the calculations showed that the low basal rate of extracellular acidification in Fig. 3A was caused entirely by production of bicarbonate, not lactate. Notably, if cells were oxidizing glucose and fats (derived, for example, from autophagy) instead of glycogen (respiratory quotient 0.6 as compared with 1), the assumption of full oxidation of glycogen would have led to negative $PPR_{glyc}$ values. $PPR_{glyc}$ was close to zero, validating our assumption. Most of the ATP was produced by oxidative reactions (oxidative phosphorylation plus substrate-linked phosphorylation in the tricarboxylic acid cycle), with only a small contribution from glycolysis (conversion of glycogen to pyruvate that was subsequently converted to bicarbonate, not lactate). The total rate of ATP production was 41.7 pmol of ATP/min/μg of cellular protein.

Upon the addition of glucose to provide exogenous substrate, the cells began to run glycolysis to lactate, leading to a 32% increase in the total ATP production rate to 55.2 pmol of ATP/min/μg of cellular protein. This is equivalent to about 0.2 fmol of ATP/s/cell. At the same time, oxidative ATP production decreased by 19.6% (from 38.3 to 30.8 pmol/min/μg of protein). Presumably, the extra ATP production by glycolysis increased the cellular phosphorylation potential, partially suppressing oxidative ATP production by the Crabtree effect and recruiting extra ATP demand to give new, higher, steady-state rates of ATP production and demand. The increase in lactate production increased the extracellular acidification rate substantially, but this effect was partially masked by a small decrease in bicarbonate production, which had been high in the basal state. The resulting change in ECAR of about 2.8-fold shown in Fig. 3A (0.94 with glucose/0.34 basal) reveals the weakness of using ECAR as a direct measure of glycolysis, because it would greatly underestimate the true change of about 7.3-fold in the glycolytic ATP production rate (Fig. 3C).

Upon the addition of oligomycin to prevent oxidative phosphorylation, the ATP demand was supplied almost entirely by glycolysis to lactate, with a tiny fraction from substrate-level phosphorylation at succinyl-CoA synthetase (SCS) accompanying the residual tricarboxylic acid cycle flux needed to drive proton leak (Fig. 3C). The increase in glycolysis caused a further increase in ECAR, which was again partially masked by the decrease in bicarbonate production (ECAR increased 1.6-fold compared with glucose (Fig. 3A), but $I_{ATPglyc}$ increased 2.1-fold (Fig. 3C). Note that this normal cellular ATP demand is insufficient to drive the glycolytic rate to its maximum capacity, which is revealed only when the demand is greatly increased (see below and see Ref. 12). These calculations illustrate the deeper insights into cellular bioenergetics that can be gained by the simple conversion of ECAR and OCR data into ATP production rates.

Fig. 3D shows the contributions of each component of glycolytic and oxidative ATP production to the total rates of ATP production shown in Fig. 3C. The relative contribution to $I_{ATPglyc}$ of ATP production by glycolysis to lactate varied from zero in the absence of added glucose (the calculated contribution was slightly negative, within the noise of the measurement) to 100% in the presence of glucose plus oligomycin, illustrating the dynamic switching between the different fates of glycolytic pyruvate as substrate supply and ATP demand were manipulated. In contrast, the relative contributions to $I_{ATPox}$ of the oxidation of glycolytic and TCA-derived reducing equivalents and substrate-level phosphorylation by SCS did not change during complete oxidation of glycogen or glucose, except when the ATP synthase was inhibited by oligomycin. This follows from the fixed stoichiometric relationship between these reactions for a given substrate (Fig. 1B).

In the supplemental material, we present these calculations as a simple Excel spreadsheet in which experimental parameters and raw ECAR and OCR data for non-growing cells metabolizing glucose may be entered on the left, and calculated values of $I_{ATPglyc}$ and $I_{ATPox}$ appear on the right.

As a worked example, this spreadsheet calculates the results underlying Fig. 3, C and D; in each well, glucose, oligomycin, and rotenone plus myxothiazol were added stepwise, and ECAR and OCR at each step were measured. The C2C12 cells were assayed at 37 °C in KRPH medium at pH 7.4 with a buffering power of 0.045 mEq/pmol of H⁺/7 μl (measured as in Ref. 5). At pH 7.4, the combined hydration/dissociation constant of CO₂ to HCO⁻₃ + H⁺ is 6.093 (5). These values are entered in cells G5–G9 under “Constants & Conditions” in the spreadsheet and are used in the subsequent calculations.

The “Data” portion of the spreadsheet is for input of raw values of protein, ECAR, and OCR data for individual wells and can be extended down as required. The worked example shows data from experiments of the type shown in Fig. 3, A and B, for a hypothetical well with the mean ECAR, OCR, and protein content of the data sets underlying Fig. 3C. The protein content of the well was 15 μg of protein (entered into cells D17–D19). Under basal conditions (medium only, with no exogenous substrate), the raw OCR was 182 pmol of O₂/min (entered into cell E17). In the presence of added glucose, OCR dropped to 158 pmol of O₂/min (cell E18), then to 62 pmol O₂/min with oligomycin (cell E19), and finally to 42 pmol of O₂/min with rotenone plus myxothiazol. To keep things simple, we assume that the rate of respiration with oligomycin (cells F17–F19) was unaffected by the addition of glucose (this could be checked and
corrected if important conclusions depended on it) and that non-mitochondrial respiration with rotenone plus myxothiazol (cells G17–G19) was a constant. The raw ECAR values were 5.2
mph/min at basal level (cell H17), 19.7 mph/min after glucose addition (cell H18), and 34.2 mph/min after oligomycin addition (cell H19).

The “Calculations” portion of the spreadsheet applies normalizations and corrections and calculates J_ATPglyc, J_ATPox, and J_ATPtot from the constants and data entered. Mitochondrial respiration per μg of protein is determined in column J,

\[
\text{J}_{\text{ATPtot}} \times \text{OCR}_m / \mu \text{g of protein} = \text{OCR}_\text{mito} \quad \text{(Eq. 1)}
\]

where \((182 - 42)/15 = 9.3 \, \text{pmol of O}_2/\text{min/μg of protein}\).

The respiration rate/μg of protein coupled to ATP production is determined in column K using a correction for oligomycin-induced hyperpolarization of the mitochondrial membrane of 0.908, taken from Ref. 15,

\[
\text{OCR}_\text{tot} - \text{OCR}_\text{coupled} / \mu \text{g of protein} = \text{OCR}_\text{coupled} \quad \text{(Eq. 2)}
\]

where \((182 - 62) \times 0.908)/15 = 7.3 \, \text{pmol of O}_2/\text{min/μg of protein}\). Total PPR/μg of protein is determined in column L,

\[
\text{ECAR}_\text{tot} / \mu \text{g of protein} = \text{PPR}_\text{tot} \quad \text{(Eq. 3)}
\]

where \((5.2/0.045)/15 = 7.7 \, \text{pmol of H}^+/\text{min/μg of protein}\). The respiratory portion of PPR tot is determined using Equation 3 from Ref. 5 and assuming a maximum H/O_2 value of 1 (see “The model system: C2C12 cells consuming glycolcyn or glucose by defined pathways”) in column M,

\[
\text{OCR}_\text{mito} \times \max \text{H}^+/\text{O}_2 \times (10^{\text{pH} - \text{pK}})/(1 + 10^{\text{pH} - \text{pK}}) \quad \text{(Eq. 4)}
\]

where \(9.3 \times 1 \times (10^{7.4 - 6.0933})(1 + 10^{7.4 - 6.0933}) = 8.9 \, \text{pmol H}^+/\text{min/μg of protein}\). The glycolytic portion of PPR is determined in column N,

\[
\text{PPR}_\text{tot} - \text{PPR}_\text{resp} = \text{PPR}_\text{glyc} \quad \text{(Eq. 5)}
\]

where \(7.7 - 8.9 = -1.2 \, \text{pmol of H}^+/\text{min/μg of protein}\). Using the OCR coupled, OCRmito, PPRresp, and PPRglyc values obtained above, rates of glycolytic and oxidative ATP production are then calculated as shown using the equations described in the text and presented in Fig. 1C. Basal J_ATPglyc is calculated using a P/O glycoly of 0.242 for glycerone (see Fig. 1B) in column O,

\[
\text{PPR}_\text{glyc} \times \text{ATP/lactate} + \text{OCR}_\text{mito} \times 2 \text{P/O glycoly} = \text{J}_{\text{ATPglyc}} \quad \text{(Eq. 6)}
\]

where \((-12 \times 1) + (9.3 \times 2 \times 0.242) = 3.3 \, \text{pmol of ATP/ min/μg of protein}\). Basal J_ATPox is calculated using a P/O oxphos of 2.486 and P/O_TCA of 0.121 in column P,

\[
\text{OCR}_\text{coupled} \times 2 \text{P/O oxphos} + \text{OCR}_\text{mito} \times 2 \text{P/O TCA} = \text{J}_{\text{ATPox}} \quad \text{(Eq. 7)}
\]

where \((7.3 \times 2 \times 2.486) + (9.3 \times 2 \times 0.121) = 38.4 \, \text{pmol of ATP/ min/μg of protein}\), assuming that in the basal state, endogenous glycerone is the primary fuel source for ATP production and that glycolytic NADH transport into the mitochondrial matrix is driven 90% by the malate-aspartate shuttle and 10% by the glycerol 3-phosphate shuttle, for an estimated P/O oxphos ratio of \((0.9 \times 2.5) + (0.1 \times 2.364) = 2.486\).

In sum, the calculations for the basal condition are 1) OCR mito = (182 - 42)/15 = 9.3 pmol of O2/μg of protein; 2) OCR coupled = ((182 - 62) \times 0.908)/15 = 7.3 pmol of O2/μg of protein; 3) PPR tot = 5.2/0.045/15 = 7.7 pmol of H+/min/μg of protein; 4) PPR resp = 9.3 \times 1 \times (10^{7.4 - 6.0933})/(1 + 10^{7.4 - 6.0933}) = 8.9 pmol of H+/min/μg of protein; 5) PPR glyc = 7.7 - 8.9 = -1.2 pmol of H+/min/μg of protein; 6) J_ATPglyc = (-1.2 \times 1) + (9.3 \times 2 \times 0.242) = 3.3 pmol of ATP/ min/μg protein; 7) J_ATPox = (7.3 \times 2 \times 2.486) + (9.3 \times 2 \times 0.121) = 38.4 pmol of ATP/ min/μg of protein.

These same calculations are applied to the measurements in the presence of glucose and glucose plus oligomycin, in both cases with the appropriate P/O glycoly value of 0.167 for glucose (see Fig. 1).

For glucose, 1) OCR mito = (158 - 42)/15 = 7.7 pmol of O2/μg of protein; 2) OCR coupled = ((158 - 62) \times 0.908)/15 = 5.8 pmol of O2/μg of protein; 3) PPR tot = 19.7/0.045/15 = 29.2 pmol of H+/min/μg; 4) PPR resp = 7.7 \times 1 \times (10^{7.4 - 6.0933})/(1 + 10^{7.4 - 6.0933}) = 7.4 pmol of H+/min/μg of protein; 5) PPR glyc = 29.2 - 7.4 = 21.8 pmol of H+/min/μg of protein; 6) J_ATPglyc = (21.8 \times 1) + (7.7 \times 2 \times 0.167) = 24.4 pmol of ATP/ min/μg of protein; 7) J_ATPox = (5.8 \times 2 \times 2.486) + (7.7 \times 2 \times 0.121) = 30.8 pmol of ATP/ min/μg of protein.

For oligomycin, 1) OCR mito = (62 - 42)/15 = 1.3 pmol of O2/μg of protein; 2) OCR coupled = ((62 - 62) \times 0.908)/15 = 0 pmol of O2/μg of protein; 3) PPR tot = 34.2/0.045/15 = 50.7 pmol of H+/min/μg of protein; 4) PPR resp = 1.3 \times 1 \times (10^{7.4 - 6.0933})/(1 + 10^{7.4 - 6.0933}) = 1.3 pmol of H+/min/μg of protein; 5) PPR glyc = 50.7 - 1.3 = 49.4 pmol of H+/min/μg of protein; 6) J_ATPglyc = (49.4 \times 1) + (1.3 \times 2 \times 0.167) = 49.8 pmol of ATP/ min/μg of protein; 7) J_ATPox = (0 \times 2 \times 2.486) + (1.3 \times 2 \times 0.121) = 0.3 pmol of ATP/ min/μg of protein.

For each condition, the total J_ATP production is calculated as J_ATPglyc + J_ATPox (column Q).

**Characterization of the cellular bioenergetic phenotype**

Describing cellular bioenergetics in terms of the total rate of ATP production (J_ATP production), divided into glycolytic (J_ATPglyc) and oxidative sources (J_ATPox) (Figs. 2A and 3C) allows quantitative and comprehensive bioenergetic analysis that is not possible with raw data. To illustrate this, a single extended data set is represented in several different ways in Figs. 4 and 5. The raw data consisted of ECAR and OCR values for C2C12 myoblasts compiled from Fig. 3 and different portions of assays for cell respiratory control (exemplified in Refs. 5 and 8) and glycolytic capacity (exemplified in Ref. 12). For each assay, we took basal measurements with no exogenous substrate, followed by measurements after the addition of 10 mM glucose. For the cell respiratory control assay, measurements continued after sequential additions of 2 μg/ml oligomycin and 1 μM FCCP (and of rotenone plus myxothiazol; not shown here). For the glycolytic capacity assay, measurements after glucose addition
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Figure 4. Visualization of bioenergetic phenotypes. A single raw data set from C2C12 myoblasts is presented in different ways in different panels. A, column plot of raw ECAR and OCR values plotted on the same y axis, with no exogenous substrate (basal) and in the presence of different combinations of glucose (g), oligomycin (o), FCCP (F), rotenone plus myxothiazol (r/m), and monensin (mon) as shown. B, data from A plotted as points with (ECAR, OCR) as (x, y) values. C, data from A converted to $J_{\text{ATPglyc}}$ and $J_{\text{ATPox}}$. D, data from C presented as stacked columns summing to $J_{\text{ATPproduction}}$. E, data from C plotted as points with $(J_{\text{ATPglyc}}, J_{\text{ATPox}})$ as (x, y) values. Constructed lines through each point show slopes denoting $J_{\text{ATP}}$ proportionality (slope = 1 denotes equal $J_{\text{ATP}}$ by source) and of 1 (denoting the same $J_{\text{ATP}}$ as the point) through each of the data points. Aggregate data from multiple experiments are presented, giving n values for each condition as follows: basal = 24, glucose = 36, glucose + oligomycin = 19, glucose + oligomycin + FCCP = 12, glucose + rotenone/myxothiazol = 8, glucose + rotenone/myxothiazol + monensin = 8. Values are means ± S.E. (error bars) of n independent experiments.

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continued after the sequential additions of 1 μM rotenone plus 1 μM myxothiazol and then 20 μM monensin.

Fig. 4A shows the data set described above in a conventional column plot, typically used for comparing the raw rates of acidification or respiration under different conditions. Clarifying the relationship of the overall bioenergetics between two conditions or samples is the implied goal of the scatter plots in Fig. 4B, where pairs of ECAR and OCR values are used as (x, y) coordinates. Comparison of two such points, representing two different conditions, cell types, cell treatments, etc., can give a crude idea of how the ATP production phenotype differs between the two samples. However, it is impossible to determine from Fig. 4B which source of ATP production predominates, and in general, no clear information can be obtained because of the arbitrary scaling between ECAR and OCR, the entangled relationships of ECAR and OCR to the rates of glycolysis and oxidative phosphorylation, and the unequal proportionality of each rate to ATP production, discussed above.

In contrast, converting ECAR and OCR measurements to rates of ATP synthesis as in Fig. 3C eliminates the above problems and allows direct comparison of the rates of glycolytic and oxidative ATP production. Fig. 4C shows the calculated values of $J_{\text{ATPglyc}}$ and $J_{\text{ATPox}}$ side-by-side, allowing comparison of their relative contributions and how each changes with conditions. This is much more meaningful than the equivalent raw data plot in Fig. 4A. Fig. 4D shows $J_{\text{ATPglyc}}$ and $J_{\text{ATPox}}$ as stacked columns, allowing comparison of the summed $J_{\text{ATP}}$ production between conditions (expanding the data set shown in Fig. 3C), which is impossible to do with the raw ECAR and OCR values and difficult to do with the side-by-side representations of $J_{\text{ATPglyc}}$ and $J_{\text{ATPox}}$.

Fig. 4E shows scatter plots of $J_{\text{ATPglyc}}$ and $J_{\text{ATPox}}$, which allow bioenergetic phenotypes to be characterized by the absolute
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Figure 5. Metabolic indices. In all panels, the solid red line connects points with different values of $J_{\text{ATP,prod}}$, but the same value of $J_{\text{ATP,prod}}/J_{\text{ATP,prod}}$ as the point with added glucose (other lines through the origin indicate different values of $J_{\text{ATP,prod}}/J_{\text{ATP,prod}}$), and lines of slope $-1$ connect combinations of $J_{\text{ATP,prod}}$ and $J_{\text{ATP,ox}}$ that sum to the same value of $J_{\text{ATP,prod}}$ (iso-$J_{\text{ATP,prod}}$). A, GI = (100 × $J_{\text{ATP,glyc}}/J_{\text{ATP,prod}}$). Vertical and horizontal dotted lines, values of the ($J_{\text{ATP,prod}}$) coordinate point for C2C12 cells with glucose (GI = 44%). The thick line of slope $-1$ connects points with GI = 50% and denotes the threshold for the a primarily glycolytic cell, defined as points with GI > 50% lying within the blue shaded area, caused by a change in glycolysis, indicated by the curved arrow showing the effect of added glucose on $J_{\text{ATP,ox}}$. C, PI = GI$_{\text{condition 1}}$/GI$_{\text{condition 2}}$ caused by a change in oxidative reactions, indicated by the curved arrows showing the effects of "removal" of oligomycin (light blue) and "removal" of rotenone plus myxothiazol (dark blue) on $J_{\text{ATP,prod}}$. D, bioenergetic capacity and bioenergetic scope. Solid black lines denote the empirical (vertical) maximum for $J_{\text{ATP,prod}}$ and the theoretical (horizontal) maximum for $J_{\text{ATP,prod}}$. The dotted black line indicates $G_{\text{max}} = G_{\text{max, capacity}} = 57\%$, passing though the theoretical bioenergetic maximum $J_{\text{ATP,prod}}$ of 109 pmol of ATP/min/µg of protein. The bioenergetic scope is indicated, after scaling to correct for the change in GI needed to achieve maximum $J_{\text{ATP,prod}}$. The shaded box indicates the bioenergetic scope: all possible points of $J_{\text{ATP,prod}}$ bounded by the maximum capacities of each supply flux. Thick black arrows indicate ATP supply flexibility; thick red arrows indicate ATP demand flexibility (see "Discussion"). E, SFI = $100 \times \theta/90°$, showing the range over which ATP demand can be met by shifting $J_{\text{ATP,prod}}$ between $J_{\text{ATP,prod}}$ and $J_{\text{ATP,ox}}$. The thick arrows indicate SFI in the presence of glucose, and the thin arrow associated with $\theta_1$ indicates how SFI would increase at lower ATP turnover. The dashed arrow associated with $\theta_2$ indicates how SFI would decrease at higher ATP turnover.
positions of the points and allow the relationships between the different conditions to be characterized by the relative positions of the points. The representation of \( J_{\text{ATP production}} \) as a single \((J_{\text{ATPglyc}}/J_{\text{ATPox}}) \) point in Fig. 4E allows us to identify whether a cell is primarily glycolytic or primarily oxidative in a way that the ECAR/OCR point in Fig. 4B cannot. In Fig. 4E, C2C12 myoblasts in the basal condition were primarily oxidative, but after the addition of glucose, they produced ATP at roughly similar rates by each pathway; this important conclusion cannot be deduced from Fig. 4B. In the presence of inhibitors of oxidative phosphorylation, they were almost entirely glycolytic, as expected. The most dramatic difference between \( B \) and \( E \) of Fig. 4 is the position of the point with glucose plus oligomycin plus FCCP. This point had high uncoupled leak respiration, appearing at high OCR in Fig. 4B, but low \( J_{\text{ATPox}} \) because of the inhibition of the ATP synthase by oligomycin and the uncoupling effect of FCCP, so it appears near the \( J_{\text{ATPglyc}} \) axis on Fig. 4E, which is much more appropriate when considering bioenergetics. The slight inhibitory effect of FCCP on \( J_{\text{ATPglyc}} \) observed previously (12) is also obvious in Fig. 4, \( D \) and \( E \), because \( J_{\text{ATPglyc}} \) and \( J_{\text{ATP production}} \) decreased upon the addition of FCCP. In the presence of glucose plus oligomycin, the cells were almost entirely glycolytic, but this is less apparent from Fig. 4B, in which the non-coupled respiration driving proton leak was not subtracted, leading to the incorrect impression that the cells were still partially oxidative. In addition, by eliminating the large contribution of bicarbonate production to the extracellular acidification rate when respiration rates were high, Fig. 4E clearly shows the large increase in glycolytic rate upon the addition of mitochondrial inhibitors, which is much less obvious in the change in total ECAR in Fig. 4B (compare also the glycolytic contribution in Fig. 3C with the ECAR changes in Fig. 3A).

The bioenergetic space plot

Once ECAR and OCR data are disentangled and converted to the same units \((J_{\text{ATPglyc}} \text{ and } J_{\text{ATPox}})\), the geometric relationships revealed in the “bioenergetic space plot” (Fig. 4E) allow the following powerful observations.

First, we define a line through the origin with a slope of 1, connecting all points at which \( J_{\text{ATPglyc}} \) and \( J_{\text{ATPox}} \) are identical (ATP production is 50% glycolytic). Points below this line represent cells that derive >50% of their ATP by glycolysis, and points above the line represent cells that derive more than 50% of their ATP by oxidative reactions. It is easy to see that C2C12 cells under basal conditions or with added glucose were more oxidative than glycolytic (both points lie above the 50% line), whereas in the presence of mitochondrial poisons, they were primarily glycolytic (the points lie below the 50% line).

Second, any line connecting all points with the same total rate of ATP production, \( J_{\text{ATP production}} \), has a slope of -1. The value of \( J_{\text{ATP production}} \) is indicated by either of the axis intersections of this line. If cells are flexible in their sources of ATP supply, they can readily move along such an “iso-\( J_{\text{ATP}} \)” line when conditions change, maintaining the same rate of ATP supply by varying the proportions derived from glycolysis and oxidative phosphorylation (this underlies the idea of indices quantifying the Crabtree and Pasteur effects and the flexibility of ATP supply; see below). When oligomycin was added to C2C12 cells using glucose, they responded flexibly by increasing the glycolytic rate to almost completely compensate for the inhibition of oxidative phosphorylation; the point with oligomycin lies near the iso-\( J_{\text{ATP}} \) line for glucose.

Third, if cellular ATP turnover increases due to increases in either supply or demand, then cells move to parallel iso-\( J_{\text{ATP}} \) lines further from the origin (this underlies the idea of indexing the bioenergetic capacity of cells and, more generally, the idea of cellular bioenergetic scope; see below). It is easy to see that when glucose was added to the C2C12 cells under basal conditions, the rate of ATP production (and, since the system was in steady state, also the rate of ATP consumption) increased substantially. Similarly, the addition of monensin (to drive increased ATP demand by the \( \text{Na}^+/\text{K}^+\)-ATPase) increased the rate of ATP production; the point with glucose plus rotenone plus myxothiazol plus monensin lies on a higher iso-\( J_{\text{ATP}} \) line than the other points. Conversely, if ATP demand decreases or if cells are inflexible to changes in conditions and cannot compensate for a decreased ATP supply through one route by increasing another, then they would move to parallel iso-\( J_{\text{ATP}} \) lines closer to the origin, as seen with the addition of FCCP to the condition with glucose plus oligomycin, which directly compromises glycolytic rate, probably by acidifying the cytosol (12).

Quantifying cellular bioenergetic phenotypes

The glycolytic index and the Warburg effect—The glycolytic index describes the degree to which a cell uses glycolysis to meet its total ATP demand. Cells with chronically increased GI values exhibit a Warburg effect. This definition is related to the underlying history and current use of the term “Warburg effect,” requiring some historical explanation. In some yeast grown under certain conditions, the presence of oxygen suppresses glycolysis to ethanol and favors oxidative phosphorylation, an observation now known as the Pasteur effect (16). Because the oxygen-independent reactions of glycolysis in mammalian cells terminate in lactate rather than ethanol, the analogous expectation is that cells exposed to oxygen (e.g. in culture) should produce little lactate. The Warburg effect describes the observation by Warburg that cancers, both tumor tissue slices and Ehrlich ascites cells, produce surprising amounts of lactate relative to non-cancerous cells (17), inconsistent with the Pasteur effect. The cause of the Warburg effect was originally proposed to be the accumulation of irreversible mitochondrial damage, but such damage was subsequently shown not to be required for significant lactate production. Still, the Warburg effect continues to underlie the view that pathological processes such as cancer subvert “normal” bioenergetic regulation to favor glycolysis (and disengagement respiration), even under aerobic conditions, and that, by extension, high rates of glycolysis maintain, may report, and may be targeted to attenuate the pathologic severity of cancer. This reasoning has been extended to the hypothesis that the source of bioenergetic supply is significant as a physiological signal or regulatory mechanism under both normal and pathological conditions (e.g. see Refs. 18 and 19), an inversion of the observation that transcriptional regulation of metabolic enzymes is
frequently altered in cancer (20). If these ideas are true, the presence of a Warburg effect in a cell should be enormously informative. However, despite an early proposal by Warburg to quantify his observations (using the Meyerhof quotient), there is currently no systematic approach for defining, quantifying, or analyzing the Warburg effect in terms of ATP turnover. In this section, we propose such an approach.

For the glycolytic index, the total ATP production rate \( J_{\text{ATP production}} = J_{\text{ATP glyc}} + J_{\text{ATP ox}} \) must be considered. We define the glycolytic index \( GI = (100 \times J_{\text{ATPglyc}} / J_{\text{ATP production}}) \). A cell whose ATP comes entirely from glycolysis to lactate therefore has a glycolytic index of 100%; a cell whose ATP comes entirely from oxidative reactions would theoretically have a GI = 0%, and a cell whose ATP comes equally from ATP\_glyc and ATP\_ox has GI = 50%. A cell is primarily glycolytic when the majority of its ATP comes from glycolysis; otherwise, it is primarily oxidative.

Fig. 5A demonstrates this characterization in the bioenergetic space plot. When provided with external glucose in aerobic culture, C2C12 myoblasts had \( J_{\text{ATPglyc}} = 24.4 \pm 1.8 \) and \( J_{\text{ATPox}} = 30.8 \pm 2.4 \), for a total of \( 55.2 \pm 3.0 \) pmol of ATP/min/\( \mu \)g of protein (mean ± S.E., \( n = 36 \)). Their glycolytic index was \( 24.4/55.2 = 44.2 \pm 6.7% \), insufficient (although not by much) to allow them to be described as primarily glycolytic. The threshold for this determination is shown as a line through the origin with slope = 1; points below this line have GI > 50% (blue shading in Fig. 5A) and describe primarily glycolytic cells; points above the line represent primarily oxidative cells.

The glycolytic index normalizes \( J_{\text{ATPglyc}} \) to \( \bar{J}_{\text{ATP production}} \), accounting for any differences in total ATP production between samples and yielding a value that can be compared with another even when the total ATP production rate is different. In addition, the normalization clarifies when the glycolytic index is unchanged between two points of comparison, which would fall on a single line through the origin even if their absolute rates of glycolysis (and therefore lactate production) were very different (all other positions on the red line in Fig. 5A).

Using the glycolytic index, the Warburg effect can be described as a chronically increased GI relative to some baseline, caused by a change in the enzymic machinery of the cell. By extension, a Warburg index (WI) can be defined as the difference between these points: \( WI = GI - GI_{\text{b}} \), where \( GI_{\text{b}} \) refers to the baseline. Importantly, Warburg’s initial observation (and conception of the eponymous effect) was based on indirect measurement of lactate production (reviewed in Ref. 21), suggesting that rapid glycolysis was required mostly for regenerating NAD\(^+\) (with glucose catabolism ending with lactate) and not for generating biosynthetic intermediates (which would not yield lactate).

The Crabtree and Pasteur indices—Unlike the Warburg effect, which describes chronic bioenergetic alteration relative to a baseline, the Crabtree and Pasteur effects reflect the acute shift of ATP supply between glycolysis and oxidative phosphorylation in response to rapid external changes, typically before and after the addition of a substrate. As with the Warburg index, the names Crabtree index and Pasteur index are deliberate reflections of the Crabtree effect and Pasteur effect, respectively, as explained below.
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change in $J_{\text{ATP,ox}}$ describes an equal and opposite change in $J_{\text{ATP,glyc}}$. Mathematically, this calculation of the Crabtree index is then the same as the value of the glycolytic index in condition 2 minus the value of the glycolytic index in condition 1 (44.2 – 8.2 = 36.0%) (Fig. 5B), so the Crabtree index is conveniently defined as $\text{Cl} = \text{GI}_{\text{condition 2}} - \text{GI}_{\text{condition 1}}$ caused by a change in glycolysis.

This Crabtree index of 36.6 illustrates that more than one-third of $J_{\text{ATP production}}$ shifted from $J_{\text{ATP,ox}}$ to $J_{\text{ATP,glyc}}$, reflecting a strong depression of oxidative ATP production by added glucose in these C2C12 cells. It reflects the underlying bioenergetics much more accurately than the simple change in oxygen consumption rate (17%) for three reasons. First, calculating the value of the Crabtree index through $J_{\text{ATP}}$ excludes non-mitochondrial respiration, which is an irrelevant confounder. Second, it can account for all ATP synthesized by the oxidative route, regardless of substrate and P/O ratios, in a way that OCR itself cannot. Third, it is unaffected by any changes in ATP turnover. In Fig. 5B, the addition of glucose increased $J_{\text{ATP production}}$ from 41.7 to 55.2 units, partially obscuring the Crabtree effect; the Crabtree index corrects for such changes. In the other extreme, a hypothetical Crabtree index of 0% would mean that even if the total ATP production rate and therefore respiration rate were to decrease upon the addition of glucose, there would be no change in the proportion of ATP derived from oxidative reactions and no Crabtree effect but simply a proportional depression of ATP turnover following the black GI = 8% line in Fig. 5B.

Theoretically, a negative Crabtree effect could occur if glycolytic activity were forced to slow (e.g. by adding an inhibitor to fully prevent glucose transport in the condition with glucose). We did not carry out such an experiment, but if it were to return all of the rates to basal, the Crabtree index would have been −36% (8–44% GI units); partial glycolytic inhibition would lead to smaller absolute values of GI.

Fig. 5C demonstrates how the Pasteur effect (the alteration in $J_{\text{ATP,ox}}$ when $J_{\text{ATP,ox}}$ is altered) can be represented in the bioenergetic space plot. In addition to the point for C2C12 myoblasts with glucose present (24.4, 30.8) shown in Fig. 5A, Fig. 5C shows the point with glucose plus oligomycin (50.0, 0.5), where GI = 99% (there is still a small $J_{\text{ATP,ox}}$ from substrate-linked phosphorylation in the tricarboxylic acid cycle even when oxidative phosphorylation is fully inhibited by oligomycin), and the point with glucose plus rotenone plus myxothiazol (47.7, 0; GI = 100%). This information can be used to quantitate the Pasteur effect as a Pasteur index.

Using the same logic as for the Crabtree index, the Pasteur index can be defined as $\text{PI} = \text{GI}_{\text{condition 1}} - \text{GI}_{\text{condition 2}}$ caused by a change in oxidative reactions. Although the Pasteur effect classically describes a decrease in $J_{\text{ATP,ox}}$ when oxygen is added to an anaerobic culture (in other words, when $J_{\text{ATP,ox}}$ is increased), it can be generalized to include a negative Pasteur effect when $J_{\text{ATP,ox}}$ under aerobic conditions is inhibited. In this spirit, the (negative) Pasteur effect can be qualitatively observed as an increase in the raw ECAR trace after the addition of oligomycin (Fig. 3A); it is qualitative because of the confounding effects of decreased respiratory acidification on raw ECAR values. Starting from the point with glucose (GI = 44%) in Fig. 5C, the addition of oligomycin moved the cells to GI = 99%, giving PI a value of 44 − 99 = −55%. Similarly, the addition of rotenone plus myxothiazol moved the cells to GI = 100%, giving PI a value of 44 − 100 = −56%. If instead we choose to consider the inhibited conditions to be the initial ones, with the experimental manipulation being the “removal” of the inhibitors, then these values change sign (the “removal” of oligomycin activates oxidative phosphorylation and gives a Pasteur index of 55%).

Indexing bioenergetic activity; bioenergetic capacity, bioenergetic scope, and the supply flexibility index—Important additional pieces of information that can be determined using this approach are the bioenergetic capacity of cells (an extension of the more limited idea of “spare respiratory capacity” (8, 25)) and the bioenergetic scope within which the cells operate, including their flexibility to respond to changes in ATP demand or to change the source of their ATP supply.

By measuring glycolytic capacity, an upper limit can be set for $J_{\text{ATP,glyc}}$. Glycolytic capacity is reached when ATP demand in the absence of oxidative ATP synthesis just exceeds $J_{\text{ATP,ox}}$. The upper limit for $J_{\text{ATP,ox}}$ is hard to achieve experimentally, because FCCP and monensin uncouple oxidative phosphorylation, and no other convenient reactions that demand high rates of ATP generation and can be turned on pharmacologically have been identified. However, as a surrogate, we can measure the maximum respiration rate in the presence of a mitochondrial uncoupler, such as FCCP, assuming that if the mitochondria were still coupled they could use this respiration to drive ATP synthesis, and treat the resulting calculated value of $J_{\text{ATP,ox}}$ as a hypothetical maximum. This assumption will fail in cells, such as brown adipocytes, in which the maximum rate of ATP synthesis is substantially less than the maximum rate of electron transport (26), but in most cells, including the C2C12 myoblasts considered here, it will probably cause little if any overestimation of the upper limit to $J_{\text{ATP,ox}}$. From the mitochondrial respiration rate in the presence of glucose + oligomycin + FCCP shown in Fig. 4A, the hypothetical maximum value of $J_{\text{ATP,ox}}$ was 46.5 pmol of ATP/min/μg of protein. However, because the mitochondria were uncoupled, the actual value of $J_{\text{ATP,ox}}$ (from substrate-linked phosphorylation in the tricarboxylic acid cycle) was 1.3 pmol of ATP/min/μg of protein, as shown in Fig. 4C.

These maximum values of $J_{\text{ATP,glyc}}$ and $J_{\text{ATP,ox}}$ define the bioenergetic capacity of the cells. As shown in Fig. 5D, the maximum individual capacities of $J_{\text{ATP,glyc}}$ and $J_{\text{ATP,ox}}$ in the bioenergetic space plot intersect at (62.5, 46.5) for a theoretical maximum bioenergetic capacity of 62.5 + 46.5 = 109.0 pmol of ATP/min/μg of protein. At this maximum point, the glycolytic index (GI_{max capacity}) would be 62.5/109 = 57.3%, making C2C12 myoblasts primarily glycolytic when running at their maximum ATP production rate. Compared with the actual
value of \( I_{\text{ATP production}} \) in the presence of glucose (55.2), the bioenergetic capacity was 109/55.2 = 197% of the rate with glucose (Fig. 5D). This bioenergetic capacity of 197% of the rate with glucose (alternatively, a reserve capacity of 109.0 – 55.2 = 53.8 pmol of ATP/min/μg of protein) reveals that the C2C12 cells under our experimental assay conditions with added glucose were operating comfortably within their capacity to generate ATP and were well set up to respond to any acute increases in ATP demand by increasing either glycolytic or oxidative ATP production, or both.

The maximum values of \( I_{\text{ATPglyc}} \) and \( I_{\text{ATPox}} \) define the full extent of the bioenergetic space that a cell can access without changing its metabolic machinery by alterations in, for example, enzyme concentration. This “bioenergetic scope” is defined by the shaded area in Fig. 5, D and E. Because it is rectilinear, the area and shape of this shaded area is fully defined by the value of the glycolytic index at maximum capacity, \( GI_{\text{max capacity}} \).

The bioenergetic scope of different cells or of the same cells treated in different ways can be compared quantitatively. For example, it is possible to say that cell X has 3.7 times the bioenergetic scope of cell Y if the area bounded by \( I_{\text{ATPglyc}} \) and \( I_{\text{ATPox}} \) is 3.7 times greater for cell X than for cell Y, regardless of the dimensions of either area. By comparing values of \( GI_{\text{max capacity}} \) it is possible to describe how different values of the maximum glycolytic and oxidative ATP production capacities allow this difference in metabolic scope.

The Crabtree and Pasteur indices quantify the response of one supply arm in Fig. 2A when the other supply arm is altered, after scaling to remove any confounding effects of changes in their sum (\( I_{\text{ATP production}} \)). A special case of this is the extent to which a cell shows flexibility to make compensatory changes in one supply pathway when the other is changed, without any such effect on \( I_{\text{ATP production}} \). A fully flexible cell could alter either supply arm to provide 0–100% of total supply (and so change its glycolytic index) without affecting \( I_{\text{ATP production}} \) whereas a less flexible cell would reach a limit beyond which it could not maintain \( I_{\text{ATP production}} \) with only a single supply arm operating. The extent to which a cell can meet a given ATP demand through either respiration or glycolysis (ATP supply flexibility) can be readily appreciated in a bioenergetic space plot. Perturbations to either \( I_{\text{ATPglyc}} \) or \( I_{\text{ATPox}} \) that do not alter total ATP demand would cause sliding of the initial point along its iso-\( I_{\text{ATP}} \) line of slope \(-1\), because the decrease in one rate was matched by an equal increase in the other, denoted by the thick black arrows in Fig. 5D. If a perturbation caused a shift that would exceed the maximum capacity of the summoned rate as shown, \( I_{\text{ATP production}} \) would necessarily decrease. The flexibility of substrate use can be captured quantitatively in the supply flexibility index, which has a value of 100% where ATP demand can be fully satisfied by any combination of \( I_{\text{ATPglyc}} \) and \( I_{\text{ATPox}} \), lower values as the excursions along lines of slope \(-1\) hit maximum capacities, and a value of 0% at the maximum ATP production capacity.

The supply flexibility index (SFI) can be defined as \( 100 \times \theta/90° \), where \( \theta \) is the angle (in degrees) defined by the lines along which a supply arm reaches its maximum capacity for the given value of \( I_{\text{ATP production}} \) as shown in Fig. 5E. For C2C12 cells with glucose, SFI was 100 × 79°/90° = 87%. This value shows that the cells had high flexibility to switch between ATP production pathways (87% of the maximum possible). The limitation was caused by the iso-\( I_{\text{ATP}} \) line leaving the area of bioenergetic scope if the cells were forced to rely solely on oxidative ATP production, although they could stay within this area if forced to rely solely on glycolytic ATP production (thick black arrows in Fig. 5E). At the slightly lower ATP demand under basal conditions (\( I_{\text{ATP production}} \) = 41.7, thin arrows in Fig. 5E), SFI would rise to 100% as the line stayed within both limits of the bioenergetic scope. At the higher hypothetical ATP demand under glucose, oligomycin, and FCCP exposure (dotted arrows in Fig. 5E), SFI would drop further and be limited by both \( I_{\text{ATPox}} \) and \( I_{\text{ATPglyc}} \) with a value of 28%. At the maximum ATP demand when the cells were at bioenergetic capacity, SFI would decrease to 0%.

### Profiling ATP consumers

To date, the major consumers of ATP generated by oxidative phosphorylation in a cell have been profiled by inferring their rates from measurements of the decrease in cellular oxygen consumption rate when individual ATP-consuming pathways are inhibited pharmacologically (27–31). However, both glycolysis and oxidative reactions generate cellular ATP that can be used by ATP consumption pathways (Fig. 6A). We have extended the previous approach (27–31) by combining it with the analysis described above, generating a complete ATP consumption profile for these C2C12 myoblasts in the presence of glucose, with both \( I_{\text{ATPglyc}} \) and \( I_{\text{ATPox}} \) accounted for. Fig. 6B shows the extent of inhibition of \( I_{\text{ATPglyc}} \) and \( I_{\text{ATPox}} \) by inhibitors of specific ATP-consuming pathways, allowing about 43% of glycolytic, 81% of oxidative, and 62% of total ATP production to be associated with specific cellular ATP-consuming processes, particularly protein synthesis and actin dynamics.

Several reports have proposed preferential consumption in a cell of locally synthesized ATP (primarily glycolytic) over the bulk ATP pool (both oxidative and glycolytic) (19, 32, 33). The idea that a spatial preference exists where ATP is consumed to 100% to make it easier to see whether any major ATP consumer has a marked preference for one source of ATP in preference to the other. Within the relatively large errors caused by measuring small percentage changes in ECAR and OCR, no such preference was apparent.

### Discussion

The updated theoretical maximum yields of ATP shown in Fig. 1A provide a summary of the current consensus ATP/glucose and P/O ratios by mitochondria and cells catabolizing different substrates. These values are of wide significance for biochemistry, physiology, and ecology and should replace the older estimates still found in some reviews and textbooks.

We describe a method for converting extracellular measurements of proton production and oxygen consumption rates (Fig. 3, A and B) into estimated rates of ATP production from...
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Figure 6. Rates of consumption of ATP by different pathways in C2C12 myoblasts. A, steady-state rates of glycolytic ATP production \( J_{\text{ATPglyc}} \) and oxidative ATP production \( J_{\text{ATPox}} \) sum to the total rate of ATP production \( J_{\text{ATPproduction}} \) and hence to the total rate of ATP consumption by multiple ATP consumers \( J_{\text{ATPconsumption}} \). ATP production and consumption are connected by ATP (or other linked variables, such as ATP/ADP or phosphorylation potential) as the common intermediate. B, absolute \( J_{\text{ATPglyc}} \) and \( J_{\text{ATPox}} \) supplying individual ATP consumers, calculated from the decrease in ECAR and OCR caused by the addition of different inhibitors of specific ATP consumption pathways (colored bars) or unassigned (by difference from the total \( J_{\text{ATPconsumption}} \) in the absence of inhibitors). The third bar shows the set of values for \( J_{\text{ATPglyc}} \) stacked above the set of values for \( J_{\text{ATPox}} \) to display total assigned and unassigned rates of ATP consumption. C, data from B scaled to the uninhibited totals for \( J_{\text{ATPglyc}} \), \( J_{\text{ATPox}} \) and \( J_{\text{ATPconsumption}} \) as 100%. No significant differences were found by unpaired t test between percentage contribution of \( J_{\text{ATPglyc}} \) and percentage contribution of \( J_{\text{ATPox}} \) for any single consumer or for “unassigned” percentage contribution of \( J_{\text{ATP}} \). The third bar shows the individual values for \( J_{\text{ATPglyc}} \) stacked above the corresponding individual values for \( J_{\text{ATPox}} \) to emphasize the contributions of each ATP consumption pathway to \( J_{\text{ATPconsumption}} \). Compounds used to define individual pathways of ATP consumption were as follows: 10 \( \mu \)M cycloheximide (protein synthesis), 25 \( \mu \)M MG132 (26S proteasome activity), 1 mm ouabain (plasma membrane Na⁺ cycling), 1 \( \mu \)M nocodazole (tubulin dynamics), 0.5 \( \mu \)M thapsigargin (intracellular Ca²⁺ cycling), and 0.25 \( \mu \)M latrunculin A (actin dynamics). Values are means ± S.E. (error bars) of \( n = 6–8 \) independent experiments/compound.

glycolysis and oxidative phosphorylation using these values (Fig. 3C). This method improves upon previous approaches in several ways. It accounts for the entire ATP turnover in the cell, not just the part linked to respiration, and it resolves technical issues related to interpreting the extracellular measurements that allow their use in a fully quantitative, rather than qualitative or semiquantitative, way. To keep things simple, the calculations here are restricted to the simple pathways of glucose and glycogen metabolism outlined in Fig. 2. However, if the rates and pathways of metabolism of other substrates or substrate mixes are known or can be reasonably assumed, in principle, the method is fully extendable to more complicated situations, such as cells growing in culture medium.

The definition of glycolytic ATP used here is biochemically sound and is the most correct way to quantify the rate of glycolytic ATP production. However, there is often great interest in knowing the rates of cellular ATP production from the anaerobic reactions of glycolysis terminating in lactate production. This provides information about how much ATP a cell can generate under anaerobic conditions and also how much lactate-linked ATP production may occur under aerobic conditions. As shown in Fig. 3, in C2C12 myocytes, pyruvate derived from exogenous glucose primarily terminates with lactate; a much smaller proportion (with much greater ATP-generating yield) is oxidized. Although it is sometimes helpful to know the rate of ATP production associated with lactate production, and this is readily calculated using our analysis, a much richer picture is obtained by the more complete analysis described here.

Once the rates of ATP production are measured, we show how a bioenergetic space plot (Fig. 4E) can reveal many aspects of the rates and flexibility of cellular bioenergetics. In particular, we have proposed indices that describe different bioenergetic phenomena using the same glycolytic index units (Fig. 5A). These units offer a unified and quantitative way to describe the bioenergetic status and flexibility of a cell, including its acute supply flexibility (The Crabtree and Pasteur indices; Fig. 5, B and C) and its capacities of total \( J_{\text{ATPproduction}} \) (bioenergetic capacity; Fig. 5D) and of supply flexibility (supply flexibility index; Fig. 5E). The proposed analysis method and tools extend or complement several existing models for conceptualizing and quantifying bioenergetic states, including cell respiratory con-
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**Cells**

Mouse C2C12 myoblasts (44) (ATCC) were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose and 2 mM glutamine, with added 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. 24 h before the assay, cells were plated in 100 μl of culture medium at 25,000-30,000 cells/well in a 24-well polystyrene Seahorse V7-PS Flux plate with no additional coating. 25 min before the assay, cells were washed three times with and then incubated in 500 μl of Krebs-Ringer phosphate HEPES (KRPH) medium (2 mM HEPES, 136 mM NaCl, 2 mM NaH2PO4, 3.7 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 0.1% (w/v) fatty-acid-free bovine serum albumin, pH 7.4 at 37 °C).

**Seahorse XF assays**

At the start of the assay, medium was replaced with 500 μl of fresh KRPH. Cell respiratory control (8) and associated extracellular acidification were assayed in a Seahorse XF-24 extracellular flux analyzer by the addition via ports A–D of 10 mM glucose, 2 μg/ml oligomycin, 1 μM FCCP, and 1 μM rotenone with 1 μM myxothiazol. For assaying glycolytic capacity, the additions (ports A–C) were 10 mM glucose, 1 μM rotenone with 1 μM myxothiazol, and 20 μM monensin. For isolating ATP consumers, the additions (ports A–D) were 10 mM glucose, vehicle or inhibitor (see Fig. 6), 2 μg/ml oligomycin, and 1 μM rotenone with 1 μM myxothiazol. All inhibitors and ionophores were prepared in DMSO or water. Two or three measurement cycles of 1-min mix, 1-min wait, and 3-min measure were carried out during each phase of the experiment. Each measurement cycle is represented by a time point in the raw trace data in the figures. Following the assay, Seahorse plate wells were washed three times with 250 μl of bovine serum albumin-free KRPH. 25 μl of radioimmune precipitation assay lysis medium (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS, pH 7.4, at 22 °C) was added. Plates were incubated on ice for 30 min and agitated on a plate shaker at 1200 rpm for 5 min. The protein concentration in each well (typically 10–15 μg/well) was measured by a BCA assay according to the manufacturer's instructions for a 96-well format assay (Thermo). Rates of oxygen consumption and extracellular acidification were expressed relative to the protein content of the appropriate well.

**Analysis of XF measurements**

The raw values of ECAR and OCR were subdivided into component rates (5). Briefly, for ECAR, the total rate of change of pH was first converted to total proton production rate (PPRtot) and then divided into proton production rates originating from respiratory bicarbonate production (PPRresp) (using OCR data) and glycolytic lactate production (PPRglyc). For OCR, mitochondrial oxygen consumption rate (OCRmito) was defined as total oxygen consumption rate (OCRtot) minus the oxygen consumption rate (OCRresp) in the presence of the respiratory chain poisons rotenone and myxothiazol (OCRmito = OCRtot – OCRresp), and the phosphorylating or coupled rate was defined as the total oxygen consumption rate minus the oligomycin-insensitive oxygen consumption rate (OCRals), with a small additional correction by 9.2% to compensate for changes in

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**Experimental procedures**

**Reagents**

Chemicals were from Sigma. Cell culture reagents and consumables were from Corning. Seahorse XF consumables were from Agilent. The bicinchoninic (BCA) protein assay was from Thermo.

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**References**

1. **Experimental procedures**

2. **Reagents**

3. **Cells**

4. **Seahorse XF assays**

5. **Analysis of XF measurements**
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mitochondrial protonotive force upon the addition of oligomycin (5) (see supplemental Table 1). Thus, OCRcoupled = 0.908 × (OCRtot − OCRoild).

The measured buffering power of KRP medium (typically used with cells) is the same when determined using a glass pH electrode or the pH sensor in a Seahorse assay cartridge (4). However, despite our earlier assertion, we found that the Seahorse pH sensor detected only about 77% of the change measured by a glass pH electrode in MAS-1 medium (45) (typical for assaying isolated mitochondria). Therefore, the buffering power of MAS-1 and possibly other media of high osmotic and/or low ionic strength should be measured using the same instrument as the experiment.

Author contributions—S. A. M. and M. D. B. conceived the project; S. A. M. carried out all experiments. S. A. M. and M. D. B. wrote the manuscript. A. A. G. and D. G. N. contributed to the development and direction of the project and reviewed the manuscript.

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