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Review

# Rethinking metabolic control

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ABSTRACT

Modulation of metabolic fluxes in plants is usually not a successful business. The main reason is our limited understanding of metabolic plasticity and metabolic control, with the latter still largely influenced by the idea that each pathway has a rate limiting step controlling the flux. Not only is experimental evidence for such steps lacking for most pathways, despite intensive search, but there are also theoretical arguments against the idea that highly regulated enzymes catalyzing reactions far from equilibrium must be considered *a priori* rate limiting. Conversely, it is argued that reactions close to equilibrium need a lot of enzyme to be maintained close to equilibrium and, contrary to accepted wisdom, begin to limit flux when reduced. Using a few key examples of plant metabolic pathways as case studies, I draw some general conclusions. The approach of augmenting flux by pushing a pathway from above is well exemplified by the attempts at increasing starch content in potato tubers, where several different approaches failed. Also pulling at the other end (close to the end product) has yielded little improvement, while targeting a reaction close to equilibrium (ADP/ATP translocation at the plastid envelope) successfully increased starch content. Rethinking control is equally well applicable to photosynthesis, with prime examples of 'neglected', unregulated enzymes exerting significant control and overprized 'limiting' enzymes having little control in normal conditions like rubisco.

In this new paradigm, the role of most control mechanisms is also challenged: feedback inhibition and post-translational modification of enzymes are relevant to metabolite homeostasis rather than flux control, with moiety conservation being a major reason for this constraint. I advocate a more extensive use of control circuitry elements (e.g. sensors like riboswitches), metabolic shortcuts and transcription factors in metabolic engineering.



**Contents**



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*Abbreviations:* G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; AGPase, ADP-glucose pyrophosphorylase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; UGPase, UDP-glucose pyrophosphorylase; PFK, fructose6P-1-kinase; SPS, sucrose phosphate synthase; FBPase, fructose 1,6 bisphosphatase; GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PRK, phosphoribulokinase; 3PGA, 3-phosphoglyceric acid; TPI, triose phosphate isomerase.

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As Mark Twain observed, while marveling at our amazing adaptation: 'Our legs are just long enough to reach the ground.' (originally cited in [1]).

## 1. Introduction

Open your favorite biochemistry textbook and count how many times is the definition 'rate limiting step' attributed to specific metabolic steps, or how often allosterically regulated enzymes are defined as "the committed step of the pathway". If a step is rate limiting, one would expect overexpression of the corresponding enzyme to give a flux increase proportional to the increase in expression. You will be disappointed to discover that such cases are extremely rare and there is, at best, a modest flux increase, way below the expected. The discrepancy between theory and practice cries out for explanation and the purpose of this review is to deal with it, summarizing research done by others over the past three decades.

Substantial flux or concentration changes are major goals for metabolic engineering, but such goals are often achieved by serendipity rather than by alteration of predicted 'controlling' enzymes. Manipulation of flux is indeed "an asymmetric problem" [2] since to stop (or to substantially reduce) a flux is enough to block (to diminish) any of the enzymatic steps in a pathway. Conversely, augmenting a flux might be problematic since overexpression of a single enzyme is often unable to bring about a (measurable) flux increase [2–5].

The rational alteration of metabolism requires a conceptual revolution in the way metabolic networks are usually considered. I will expose some of the misunderstandings hampering the revolution by recalling paradigmatic examples of failed plant metabolic engineering. The conclusions are however not limited to the plant field and the specific examples, but can be applied to metabolism in general. Due to my limited knowledge of the animal and microbial world, others, with better understanding of these fields, will fill in the gaps, as chanted by Dante: "A little spark is followed by great flame/Perchance with better voices after me/ Shall prayer be made that Cyrrha may respond!" (The Divine Comedy, Paradise, Canto I, v 34–36). Readers uninterested in plant metabolism may skip the next section.

## 2. Starch, more or less?

A paradigmatic example of experiments yielding results opposite to those expected is the manipulation of sugar metabolism in the attempt to increase starch content in potato. Several papers reported investigation for most of the enzymes involved in starch metabolism by under- or over-expression of endogenous genes. In some cases genes have been overexpressed in different compartments of potato tubers or new enzymatic specificities were introduced, such as sucrose phosphorylase, bacterial xylose isomerase and glucokinase. The work has been extensively reviewed [6–9]. A decrease in starch yield is of course expected, and obtained, in antisense lines for enzymes in the pathway to starch, e.g. ADP-glucose pyrophosphorylase (AGPase), plastid and cytosol phosphoglucomutase (PGM), starch synthases and so on. Usually their flux control coefficient ($C^J$, a measure of the degree of flux control by an enzyme, see [10] for a detailed introduction to Metabolic Control Analysis and its jargon) is small, meaning that a reduction in each of these enzymes does not cause a proportional reduction in flux. Conversely, the overexpression of enzymes like glucokinase, cytosolic invertase or sucrose phosphorylase, whose products are either a sugar (glucose, fructose) or a phosphorylated sugar in the pathway to starch, was expected to increase the concentration of free or phosphorylated sugars and the flux to starch. These manipulations fit into the strategy of 'push-from-above' (with similarity to forced goose feeding) by supplying more precursors to the tuber starch pathway). Some of the reactions forming the early precursors fructose, glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) were considered, for some apparently self-evident reason, good targets for metabolic engineering strategies. Several of these early steps are reversible and close to equilibrium *in vivo*, like sucrose synthase and UDP-glucose pyrophosphorylase (UGPase). For instance, the enzymatic activity for UGPase is 600 times the net flux going through this reaction [11]. Therefore expressing alternative enzymes, e.g. invertase (catalyzing an irreversible reaction) or sucrose phosphorylase instead of sucrose synthase, for sucrose cleavage was expected to increase sink strength and starch accumulation. The substitution of a reversible step with an irreversible one was indeed considered the way to augment flux in the whole pathway.

Invariably, these manipulations resulted in increased respiration, inhibition of starch accumulation and a decreased starch yield [12–15] (see also [16] for a milder phenotype). The above mentioned transgenes were also combined among themselves or with other transgenes like antisense AGPase, but the result was usually one and the same: less starch and more glycolysis. For instance, overexpression of invertase in the cytosol was accompanied by a drop in sucrose and an accumulation of glucose [12]; therefore a bacterial glucokinase "free of any regulatory or sensory properties" was added to increase glucose phosphorylating capacity and move glucose down the pathway, the result being a further reduction in starch, a large increase in phosphorylated sugars and an increase in glycolysis and respiration [13]. Similar result was obtained with a bacterial sucrose phosphorylase [15]. Therefore sucrose hydrolysis is not limiting, neither is hexose phosphorylation, as suggested also by antisense repression of hexokinase1 and 2 [17,18], nor is sugar transport at the plasma membrane [19].

Working at the other side of the pathway with the approach of 'pulling-from-below' (drain intermediates into starch by forcing the terminal reactions) did not fare better and early reports of increased starch content due to overexpression of a bacterial AGPase with reduced allosteric inhibition [20] could not be replicated by another group [21]. Similar attempts in cassava did not increase starch content, even though total plant biomass increased [22]. In wheat and rice, the gain in seed weight per plant in transgenics carrying an altered maize AGPase large subunit was mainly the result of increases in heads per plant and in the number of seeds per head [23,24]. Tampering with the adenylate [25] or the pyrimidine pool [26] or overexpression of a regulator kinase [27] are effective means of increasing the starch content of potato, even though the mechanism through which this is achieved is unclear. What is then limiting starch accumulation? One expected that, according to current views, the control of flux resides in reactions with large, negative $\Delta G$. Comparing these with flux control coefficients for starch biosynthesis (Fig. 1 and Table 1), it is clear that the highest $C^J$ is associated with the adenylate transporter of the plastid envelope [28–30,6]. Similar results, albeit not identical, were obtained in potato by expressing a G6P/phosphate translocator from pea together with an adenylate translocator from *Arabidopsis* [31].

Indeed, the adenylate transporter, was hardly imagined to play a key role in starch synthesis. It catalyses a reaction with $K'_{eq}$ equal to 1, that is $\Delta G^{\circ\prime} = 0$, because when the concentrations of adenylates are all equal to 1 M there is no net flux among the compartments, assuming a negligible plastidial transmembrane potential, which is probably the case [32]. The ATP flux into the plastid and the flux to starch, which consumes ATP for producing ADP-G, are thus highly sensitive to changes in the amount of

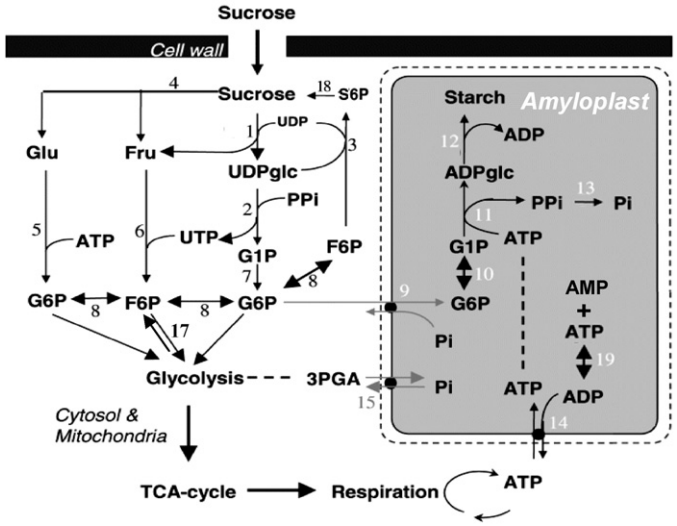


**Fig. 1.** Starch synthesis: flux control distribution and thermodynamic parameters. A schematic representation of major metabolic steps involved in starch biosynthesis is presented (redrawn from [6]). Each reaction in the scheme is identified by a number and listed in Table 1 with the name of the corresponding enzyme, its flux control coefficient, mass action ratio (Q) and equilibrium constant ($K'_{eq}$), when available. Reaction data are from [34] while $C^J$s are from [6]. The $K'_{eq}$ value for sucrose 6-phosphate hydrolysis was assumed to be the same as for glucose 6-phosphate, for pyrophosphate dependent-phosphofructokinase (PFP) was from [124] and for sucrose phosphate synthase from [125]. The phosphoglucoisomerase reaction is defined in reverse direction as in [34] and in accordance with Table 2 (G6P as substrate).

transporter. The adenylate transporter of potato tubers is a true example of a single enzyme being rate limiting, but it catalyzes a reaction with little regulation and whose energy change, both standard and *in vivo*, is close to 0.

## 3. Challenging the dogma

### 3.1. An often repeated adagio about irreversible reactions

Classical biochemistry claims that reactions working close to equilibrium are usually not regulated, which is true, and cannot regulate flux. Conversely, an old adagio of textbooks is that the control of flux resides in reactions with large negative changes in Gibbs energy. These concepts keep being repeated, but without the support of experimental evidence. I challenge this belief on logical grounds as well as on experimental evidence. Readers unfamiliar with the vocabulary of thermodynamics are referred to Box 1 for a brief summary of definitions and relationships.z

The Gibbs energy variation in standard conditions ($\Delta G^{\circ\prime}$) for any reaction is a constant at constant temperature. The actual variation in Gibbs energy ($\Delta G$) for the same reaction can be significantly different from $\Delta G^{\circ\prime}$ and depends on reaction conditions, that is the activity (usually approximated to the concentration) of reactants. It is incorrect to use $\Delta G^{\circ\prime}$ instead of $\Delta G$ to predict the direction and the degree of spontaneity of a reaction in experimental conditions, but let me suggest some 'rules of thumb'. If a reaction has the same number of substrates and products, like PGM, phosphoglucoisomerase (PGI), UGPase or fructose6P-1-kinase (PFK), then its $\Delta G$ *in vivo* will not usually differ from $\Delta G^{\circ\prime}$ by more than a few kJ/mol [33,11,34]. Keep in mind that when a reactant is at constant concentration, like water or protons, its value is incorporated in the $K'_{eq}$. For the present discussion then, reactions like G6P hydrolysis to Glucose + P must be considered as a one substrate/two products reaction. An example of a reaction with equal number of substrates/products is G6P ↔ F6P catalyzed by PGI; it has a $\Delta G^{\circ\prime}$ of +1.7 kJ/mol and, in the cytosol of potato tubers, a $\Delta G$ of −1.38 kJ/mol (see Table 2 for a summary of the different parameters for some reactions mentioned). This is because most metabolites are in the 0.1–1 mM range in the cell and this makes the reactants' ratio (Q) similar to the ratio in standard conditions (obviously equal to 1). For PGI, Q in potato tuber is: [F6P]/

**Box 1.** Thermodynamic definitions, relationships and conversions

Definitions

| | |
|---|---|
| $\Delta G$ | Gibbs energy change in experimental conditions |
| $\Delta G^{\circ}$ | Gibbs energy change in standard conditions (reactants all 1 M) |
| $\Delta G^{\circ\prime}$ | Gibbs energy change in standard conditions for biochemistry (as before, but pH 7) |
| $K'_{eq}$ | Equilibrium constant of the reaction in standard conditions for biochemistry |
| $Q$ or $\Gamma$ | Mass action ratio (also known as reaction quotient): ratio of reactants' molar concentrations. For reaction: A ↔ B + C, $Q = ([B][C])/[A]$ |
| $\rho$ | Disequilibrium ratio (measures the degree of displacement from equilibrium). |

Relationships

$$\rho = \frac{Q}{K'_{eq}}$$

$$\Delta G = \Delta G^{\circ\prime} + RT\ln(Q)$$

$$\Delta G^{\circ\prime} = -RT\ln(K'_{eq})$$

$$\Delta G = \Delta G^{\circ\prime} + RT\ln(Q) = -RT\ln(K'_{eq}) + RT\ln(Q) = RT\ln\left(\frac{Q}{K'_{eq}}\right) = RT\ln(\rho)$$

Note that $\Delta G$ and $\rho$ are two different measures (the first logarithmic, the second linear) of the reaction driving force (same for $\Delta G^{\circ\prime}$ and $K'_{eq}$ in standard conditions).

At equilibrium: $Q = K'_{eq}$, $\Delta G = 0$ and $\rho = 1$

Conversion between the logarithmic and linear values

Given that $R = 8.314$ (J mol$^{-1}$ K$^{-1}$), $T = 298$ K, $\ln(x) = 2.3025 \times \lg(x)$, hence: $\Delta G = RT\ln(\rho) = 2.303RT\lg(\rho) = 2.303 \times 8.314 \times 298 \times \lg(\rho) = 5705 \times \lg(\rho)$ [expressed as J/mol]

Similarly $\Delta G^{\circ\prime} = -RT\ln(K'_{eq}) = -5705 \times \lg(K'_{eq})$ [expressed as J/mol]

A 10 fold difference in Q equals to a difference of 5705 J/mol in $\Delta G$

**Table 1**
List of reactions, and corresponding data, as identified by numbers in Fig. 1 (see figure legend for details).

| # | Enzyme | $C^J$ | Q | $K'_{eq}$ |
|---|---|---|---|---|
| 1 | Sucrose synthase | 0.10 | 0.181 | 0.15–0.56 |
| 2 | UDP glucose pyrophosphorylase | ~0.0 | 1.48 | 3.2 |
| 3 | Sucrose phosphate synthase | −0.15 | | 3250 |
| 4 | Invertase | | | 137000 |
| 5 | Hexokinase | <0.1 | | 780 |
| 6 | Fructokinase | | 0.032 | 851 |
| 7 | Cytosol phosphoglucomutase | 0.15 | 11.9 | 19 |
| 8 | Phosphoglucoisomerase | | 0.288 | 2 |
| 9 | G6P/Pi translocator | | 2.04 | 1 |
| 10 | Plastid phosphoglucomutase | 0.23 | 0.046 | 0.053 |
| 11 | ADP glucose pyrophosphorylase | 0.35 | 0.016 | 1 |
| 12 | Starch synthase | 0.12 | | |
| 13 | Alkaline pyrophosphatase | | 0.323 | 1000 |
| 14 | Adenylate translocator | 0.98 | 0.359 | 1 |
| 15 | 3PGA/Pi translocator | | 1.85 | 1 |
| 16 | Starch branching Enzyme | ~0.0 | | |
| 17 | Pyrophosphate-dependent PFK (PFP) | 0.25 | | 3.2 |
| 18 | Sucrose-6phosphate phosphatase | | | 264 |
| 19 | Plastid adenylate kinase | −0.75 | | 1 |

**Table 2**
Thermodynamic parameters for some enzymatic reactions.

| Reaction | Enzyme | $\Delta G^{\circ\prime}$ (kJ/mol) | $\Delta G$ (kJ/mol) | $K'_{eq}$ | $Q$ | $\rho(Q/K'_{eq})$ |
|---|---|---|---|---|---|---|
| G6P ↔ F6P | PGI | +1.70 | −1.38 | 2.0 | 0.288 | 0.144 |
| F1,6BP ↔ DHAP + GAP | Aldolase | +23.8 | −1.7 | $6.73 \times 10^{-5}$ M | $1.33 \times 10^{-4}$ M | 1.98 |
| F6P + ATP ↔ F1,6BP + ADP | PFK | −14.2 | −22 | 310 | 0.0432 | $1.4 \times 10^{-4}$ |
| 1,3BPGA+ADP ↔ ATP + 3PGA | 3PGA kinase | −18.9 | +0.1 | 2060 | 2150 | 1.04 |
| ATP → ADP + P | – | −30.5 | −47.5 | $2.22 \times 10^{5}$ M | $10^{-3}$ M | $2.11 \times 10^{-8}$ |
| RuBP + CO2 → 2 (3PGA) | Rubisco | −35.1 | −41.0 | $1.42 \times 10^{6}$ | $9.24 \cdot 10^{-2}$ | $6.51 \times 10^{-8}$ |

$\Delta G$, $Q$ and $\rho$ of phosphoglucoisomerase (PGI), aldolase and ATP hydrolysis are calculated for the conditions in potato tubers [34,47]. Values for PFK, 3PGA kinase and Rubisco are found in most biochemistry textbooks for physiological conditions.

[G6P] = $1.47 \times 10^{-4}$ M/$5.10 \times 10^{-4}$ M = 0.288. Similar consideration can be made for triose phosphate isomerase (TPI), enolase and PGM, other enzymes in glucose metabolism. When one or more reactants on the same side of the equation have very different concentration values from the others, e.g. in the low μM range, $Q$ may differ more substantially, even by a factor of 1000 (e.g. 3PGA kinase). The same is true for reactions with unequal numbers of substrates vs. products [35]. This means that $\Delta G$ could differ even by >20 kJ/mol from $\Delta G^{\circ\prime}$. For instance, aldolase, which catalyses also the reaction F1,6BP ↔ DHAP + GAP, has $\Delta G^{\circ\prime}$ = +23.8, corresponding to a $K'_{eq} = 6.73 \times 10^{-5}$ M. Yet the reaction *in vivo* is close to equilibrium ($\Delta G$ = −1.7 kJ/mol) [35]. Another example is ATP hydrolysis: its $\Delta G^{\circ\prime}$ is −30.5 kJ/mol, but ATP, ADP and inorganic phosphate ($P_i$) are all in the 100 μM to 1 mM range *in vivo* and thus $Q$ is around $10^{-3}$. The uncertainty over actual concentration values (ADP and Pi found by extraction methods are usually higher than those obtained by $^{31}$P NMR spectroscopy, see [36,37]) does not undermine the argument, since $\Delta G$ is in both cases more negative than $\Delta G^{\circ\prime}$ by at least 17 kJ/mol.

In summary, if a reaction has a very large negative $\Delta G^{\circ\prime}$, it cannot usually be allowed to go to equilibrium. Otherwise substrates would be quantitatively converted to products, with very little substrates remaining just to satisfy the equilibrium conditions. Consider the reaction catalyzed by PFK: F6P + ATP ↔ F1,6BP + ADP, having a $K'_{eq} = 310$ ($\Delta G^{\circ\prime}$ = −14.2 kJ/mol) and an *in vivo* $\rho(Q/K'_{eq})$ around $1.4 \times 10^{-4}$ ($\Delta G$ = −22 kJ/mol). Substituting the actual *in vivo* ATP and ADP concentrations [34] and allowing the enzyme to run unrestricted, then the product F1,6BP would become around 1000 times more concentrated than F6P, which is around 0.15 mM in the cytosol [34], surely a problematic condition. Allowing reactions like hexokinase, PFK or fructose 1,6 bisphosphatase (FBPase) to run wild would cause the build up of unbearably high concentrations of certain metabolites or vanishing low concentrations of other ones, with clear repercussions on other pathways departing from these metabolites. This can be understood using the analogy between metabolic pathways and water flow among tanks placed at different heights. Allowing unrestricted flow between tanks with a large water height difference, e.g. 'reaction' A to B or C to D in Fig. 2, will only cause the filling in of 'product' tanks (B and D, respectively) and a very low level in tanks A and C. The analogy is limited and wants solely to convey the importance of both driving force (height or pressure difference) and enzyme quantity (pipe cross section) in determining flux.

### 3.2. Reversible reactions: too much enzyme or just about right?

Again as a rule of thumb, reactions whose $\Delta G^{\circ\prime}$ is not far from 0, such as those catalyzed by PGI or PGM, usually must be kept close to equilibrium (the issues discussed before on the number of reactants and their actual *in vivo* concentrations must also be considered). If such reactions are prevented from approaching equilibrium by restricting enzyme activity, their substrates will accumulate because the reactions are embedded in pathways at steady state. The concentration of their products will drop with, again, effects on other pathways. When close to equilibrium, these reactions have a substantial negative term $(-P/K'_{eq})$ in the reversible Michaelis–Menten expression (see Fig. 3 legend). Because P is close to S and $K'_{eq}$ is close to 1, the actual v becomes much smaller than $V_{max}$. The only way to have these reactions near equilibrium and a flux high enough is to have a large $V_{max}$ compared to the actual pathway flux. The same $(-P/K'_{eq})$ term tends to be negligible for reactions with large $K'_{eq}$. This is easily appreciated from Fig. 3. The four curves are the enzyme velocities as a function of substrate concentration calculated at four different product concentrations. Assuming substrate concentration is fixed (vertical dashed line) and increasing product concentrations, the ratio between the velocities at the lowest (0) or at high (5) product concentration is very large: 150 fold. Unconvinced readers should create another set of curves using a value of 1000 for $K'_{eq}$. It is unfortunate that the irreversible Michaelis–Menten equation is the standard, and often the sole, kinetic law taught in biochemistry courses to describe enzyme behavior. Even when more complex kinetics are treated for describing reactions with multiple

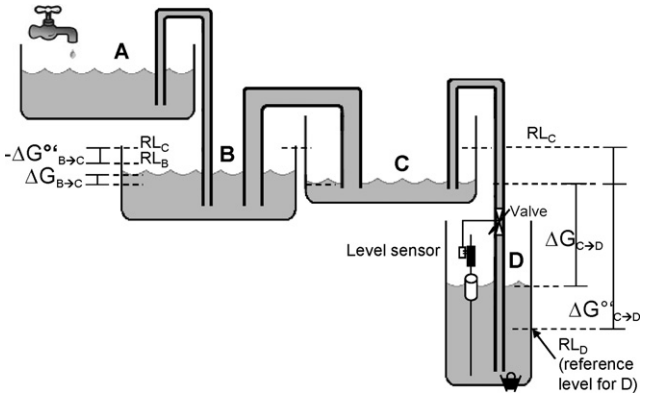


**Fig. 2.** The law of the flush: water flow depends on both driving force and pipe carrying capacity. Four tanks (A to D) represent metabolite pools and siphons connecting tanks represent enzymes converting one metabolite into the other. The maximal flux carrying capacity is the pipe cross section. The actual flow in a pipe (actual enzyme velocity v for a metabolic reaction) depends on (1) the driving force, i.e. the height difference in the water levels between two tanks ($\Delta G$ for reactions) and (2) the pipe cross section ($V_{max}$, proportional to enzyme quantity). When the driving force is large (large height difference as in the A to B or C to D reactions), then regulating mechanisms, such as a device measuring water level in tank D (tank level sensor) controlling a valve on the siphon, must be in place. When reactions have a small driving force (e.g. B to C), a large flux carrying capacity, but no sensor-valve couple, is required. Note that the driving force is linearly dependent on the height difference, while on the chemical level the driving force ($\Delta G$) depends on the logarithm of the concentration ratio. The $\Delta G^{\circ\prime}$ could be interpreted in the analogy as the height difference between tanks when the water in each tank is at a reference level (RL) corresponding to a unitary height (as 1 M is the reference for reactants). In the B to C reaction, flow would be reversed in the reference condition due to a higher level of water in tank C. A source (tap above tank A) and a drain (bottom of tank D) are depicted for completion. The figure does not pretend at all to capture the intricacies of metabolic pathways with multiple substrates and products, coupled reactions and moiety conservation.

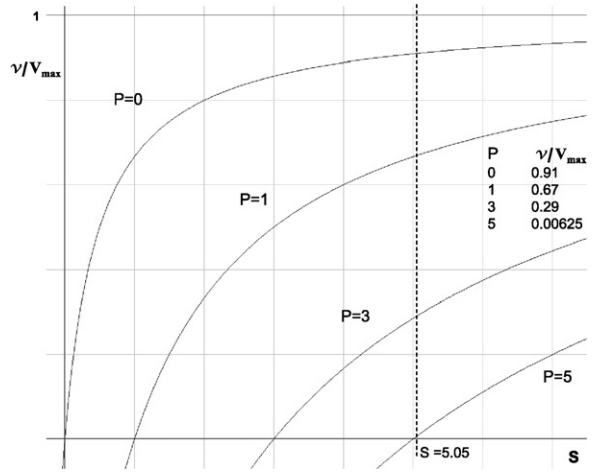


**Fig. 3.** Enzyme velocity for an enzyme catalyzing a reaction close to equilibrium is strongly dependent on $\rho$ and can be orders of magnitude smaller than $V_{max}$. Velocity vs. substrate concentration plots for a reversible 'Michaelis–Menten' enzyme at four different product concentrations ($P = 0$, 1, 3 and 5). Enzyme rate law: $v = (V_{max}/K_s)(S - P/K'_{eq})/(1 + S/K_s + P/K_p)$, with following parameters value: $V_{max} = 5$, $K_s = 0.5$, $K'_{eq} = 1$, $K_p = 1$. Actual enzyme velocity ($v$) is calculated from the rate law using the parameters and $S$ and $P$ values.

substrates and/or products, it is always the irreversible version which is employed, because it is assumed that the products are always at negligible concentration, a situation valid only *in vitro*.

Again using the water tank analogy (Fig. 2), a sustained flow between tanks B and C is only possible by having a large pipe connecting the two tanks, because the driving force is small: flow in ideal pipes is proportional to the pressure difference which in turn is proportional to the water height difference. When flux through the pathway decreases downstream of an enzyme working close to equilibrium, then there is no need to restrict the velocity of the enzyme: the diminished steady state flux will increase the ratio of reactants and the reaction will automatically become slower. For instance, if the pathway consuming the product is slowed down, then the product concentration will rise, moving the velocity downward along the dashed line in Fig. 3. Similar arguments can be put forward if one slows down or accelerates the supply of substrate. In other words, the regulation of flux is embedded in the thermodynamics of the reaction because it is close to equilibrium.

One relevant conclusion so far is that reactions, whose *in vivo* $\Delta G$ is large and negative, must be kept far from equilibrium and that the required maximal catalytic activity is not much larger than the *in vivo* flux. It is however *imperative* that such activity is well regulated. In order to tune the reactions with the metabolite flow of the pathway and avoid concentration excesses, there must be regulatory mechanisms, like feedback inhibition, allosteric modulators or other post-translational modification [38,39]. The second important conclusion is that, conversely, reactions close to equilibrium require a lot of enzyme, given the small driving force. In this case, the reaction rate is sensitively dependent on the driving force. When it is reduced, flux too is reduced. Indeed such enzymes often have maximal rates 100–1000 fold higher than the actual pathway flux (e.g. TPI, UGPase [10,11]). Several are so abundant that they represent a significant share of total proteins (examples are plastid PGM, transketolase, aldolase; [40]). This is the only way to guarantee the proximity to equilibrium. The fact that most of the enzyme is occupied shuttling back and forth substrates and products is not an avoidable mistake. It is the necessary price which must be paid to maintain the reaction close to equilibrium. There are several examples where a decrease in enzymes involved in reactions close to equilibrium causes

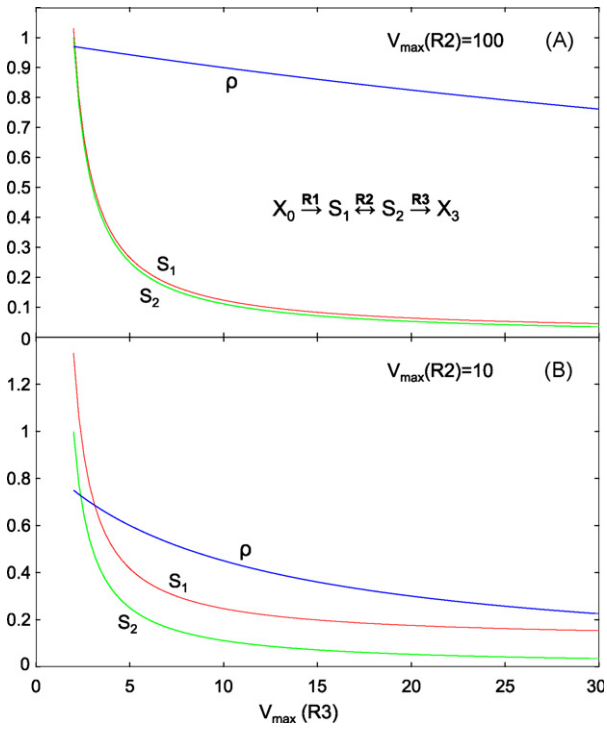


**Fig. 4.** The disequilibrium ratio of a reaction close to equilibrium is sensitive to changes in $V_{max}$. A pathway consisting of three one-substrate/one-product Michaelis–Menten reactions was modeled with GEPASI (www.gepasi.org) using the following definitions and parameters: reaction1 (R1): irreversible $X_0 \rightarrow S_1$, $V_{max} = 2$, $K_m = 1$; reaction2 (R2): reversible $S_1 \leftrightarrow S_2$, $K_{ms} = 1$, $K_{mp} = 1$, $K_{eq} = 1$, $V_{max}$ either 100 (A) or 10 (B); reaction3 (R3): irreversible $S_2 \rightarrow X_3$, $V_{max} = 2$, $K_m = 1$; $X_0$ was fixed at 1 mM. $X_3$ concentration can be fixed at any value. The steady state values for $\rho$, $S_1$ and $S_2$ are plotted as a function of the $V_{max}$ of reaction3 (varied between 2 and 40). The scheme of the pathway is presented in panel A. To appreciate a change in $V_{max}$ of reaction2, fix a value for the $x$-axis and compare $S_1$ and $\rho$ in the two conditions.

significant flux restrictions: aldolase [41,42], transketolase [43], plastid PGI [44], plastid PGM [45,46]; see also Table 1.

The sensitivity of $\rho$ to changes in $V_{max}$ can be appreciated by simulating a three reaction pathway (Fig. 4, readers allergic to modeling may skip the figure). This simple ideal metabolic system comprises a reaction catalyzed by a reversible enzyme with $K_{eq} = 1$ sandwiched between two irreversible reactions. As its $V_{max}$ is reduced 10 fold (panel A vs. panel B), the disequilibrium ratio becomes smaller (and $\Delta G$ more negative) implying that the reactions moves further away from equilibrium. This happens because $S_1$ concentration increases. Similarly, when the $V_{max}$ of the third enzyme is increased, $\rho$ decreases. Both $S_1$ and $S_2$ drop, but $S_2$ drops faster than $S_1$ (see for instance panel B in Fig. 4). The same value of $\rho$ (far right of panel A or far left of panel B) occurs with quite different metabolite concentrations. A close to equilibrium condition ($\rho$ close to 1) does not ensure that these nearly equal concentrations are low at steady state, because they are determined by the rest of the system.

Negative regulation is thus crucial and necessary for enzymes catalyzing reactions far from equilibrium. Negative regulation would be of no good for reactions close to equilibrium as this may only increase the driving force and restrict flux. For this reason it is no surprise that enzymes catalyzing reactions close to the equilibrium are not subjected to regulation. It is therefore misleading to consider regulated steps as being regulatory (see below). Despite the technical proficiency of groups investigating metabolism with transgenics and metabolomics, their choices of targets are often based on unjustified premises. What is then happening when transgenes are unsuccessfully engineered for

instance for an increased starch content? Is there some signal being released that diverts organic carbon into respiration and away from starch synthesis? The answer is 'yes and no'. Yes in the sense that such a signal exists and it is quite measurable. No, in the sense that this so called 'signal' is nothing new.

## 4. Cycles and conservation laws

Why is it so important to avoid metabolite excesses? Any increase in the concentration of a sugar has an effect on cell osmolarity, but this presumably is not a problem unless the concentration reaches very high levels, in the range of several tens of mM. Long before this can happen, another phenomenon emerges as significant for the regulation of pathways. Much reduced carbon can be converted to sugar and polymerized into compounds like starch or cell wall components. This large flux takes place at steady state because intermediates are produced and removed at the same rate. A potential problem starts arising when a substrate-phosphorylating reaction works unrestricted. As more sugar gets phosphorylated and the product accumulates, there is an immediate danger for a cell: $P_i$ insufficiency. In fact, additional carbon can be taken rapidly from the inexhaustible supply of atmospheric $CO_2$, while the amount of the phosphorylated metabolites is limited by phosphate availability in the short term, and this is not easily adjusted; intracellular free phosphate is in the mM range and the sum of Pi and all phosphorylated metabolites in the potato tuber cytosol is in the range of 10 mM [34,47] and cannot be changed quickly. Moreover $P_i$ is being used in many compounds whose concentration is a significant fraction of total P and which can be varied only slightly like ATP, ADP (and obviously all the other nucleotides), phospholipids, phosphoproteins and others.

In metabolic jargon this is termed 'moiety conservation': the sum of all phosphate contained in a cell (free phosphate, sugar phosphates and all other phosphorylated compounds) is relatively constant within the timeframe of minutes. This is not due however to the absence of turnover, but rather by an equal counterbalance of synthesis and degradation. Taking into account phosphate quantity, flux in major pathways involving consumption or production of P or P-containing compounds is large enough to be able to quickly alter the concentration of several intermediates. Every few minutes most of the sugar phosphates in a potato cell is consumed and replaced by freshly made molecules: the half life of plastidial G1P, for instance, must be less than 1 min based on the data of flux and metabolite content [47,29].

The consequences should now be clear: cells cannot afford to lock all of their phosphate into phosphorylated intermediates (sugars or acid) as would happen when reactions like PFK or sucrose phosphate synthase (SPS) run freely (i.e. approach equilibrium). For instance, overexpression of invertase causes a dramatic accumulation of sugar phosphates, but a dramatic drop in free phosphate in the cytosol [48]. Long before any osmotic problem could arise, the shortage of free phosphate or other phosphorylated metabolites would be problematic or lethal. The activities of several enzymes such as PFK, FBPase, SPS, . . ., etc., are controlled as a safety measure to prevent this. A similar phenomenon takes place with ATP metabolism. ADP and AMP should not be converted entirely to ATP, because reactions requiring ADP would come to a halt. In the 1950s the hypothesis was put forward that glycolysis would be limited by the availability of either a phosphate or electron acceptor such as ADP or $NAD^+$ [49], but this hypothesis was later discarded when direct measurements showed that these compounds were not depleted under any condition [50]. These compounds are not depleted because enzyme regulation maintains them homeostatically. For instance, small changes in ATP/ADP ratio are amplified at the level of AMP by adenylate kinase. AMP levels regulate enzyme activity either directly as an allosteric activator (e.g. PFK in plants) or indirectly through the AMP-activated protein kinase (e.g. [51,52] for examples from non-plant systems).

Metabolite homeostasis is thus important and the control of reaction rates of 'regulated' enzymes is indeed a control that maintains metabolite concentration constant, avoiding excesses. A clear example of metabolite homeostasis that has gone wrong is the manipulation of the folate pathway. By overexpressing one of the early enzymes in a pterin branch, one regarded as limiting, causes around a thousand fold accumulation of precursor pterins and a very small increase in end product [53]. This kind of effect is the most likely outcome for single enzyme manipulation [54,3,2] and the use of desensitized enzymes can only exacerbate the effect. Many such failed metabolic engineering attempts on plant carbohydrates are summarized in ref [55]. Disruption of metabolite homeostasis can also have paradoxic effects because some metabolites act as intracellular signals. For example, manipulating the glutathione metabolism by overexpression of either the first or both of the two biosynthetic enzymes significantly increased GSH content, but also caused a 10-25 fold increase of γ-glutamylcysteine [56], a significant share of which was in a more oxidized state. This enhanced $H_2O_2$ content and oxidative stress presumably due to a failure of the redox-sensing process in the chloroplast.

## 5. Virtue out of necessity?

If regulation of enzyme activity is a necessity to avoid metabolite excesses, why is it normally turned into a virtue (the capacity to regulate flux)? This confusion emerges also in the concepts 'regulated' ('highly regulated' is usually applied to enzymes modulated by factors other than substrates or products) and 'regulatory', with the former apparently implying the latter: the factors controlling/regulating enzyme activity will regulate the flux. The plea for a quantitative definition of regulatory mechanisms on metabolite concentrations and fluxes, first proposed by Hofmeyr and Cornish-Bowden in 1991 [57,58] has remained unheard. The explicit claim raised here is that regulation in the sense of post-translational modification, or allosteric interactions modulating enzyme activity, is not responsible for flux control. Rather it is relevant to metabolite concentration control, that is, metabolite homeostasis.

Most transgenics designed to increase starch make less starch because the increase in phosphorylated sugars switches on sucrose synthesis via SPS. SPS activity is stimulated by an increase in substrates (F6P and UDP-G), allosteric activator (G6P) and a decrease in inhibitor ($P_i$, [59]). In the transgenics all metabolites change in the direction of an activation of SPS, thereby priming a futile cycle of sucrose synthesis and degradation that wastes much cellular ATP [60,14]. The additional level of enzyme regulation offered by 14-3-3 proteins and phosphorylation of SPS is not enough to reduce flux through the branch [61]. It becomes clear that tampering with mechanisms for metabolite control is not the best approach, because of unforeseen (but not necessarily unforeseeable) consequences (e.g. [60]; see also below).

This does not yet answer the question of why the plastidial adenylate trasporter limits the flux to starch. The reason is that tubers become easily hypoxic and this restricts energy production. Increasing energy consumption gratuitously leads to a decrease in the ATP to ADP ratio and creates a steeper oxygen gradient within growing tubers [29]. A hypoxic tuber could run out of ATP and because of this, energy consumption in the plastid has to be tuned with energy production in mitochondria, particularly when mitochondrial ATP output is small. The low level of transporter seems to be well suited to this end, as it would restrict the flux more when the concentration gradients driving the transport are

small, i.e. the reaction is close to equilibrium. Therefore I anticipate that transgenic tubers with increased transporter should be more sensitive to hypoxia (e.g. in flood conditions) than the wild type. Trying to engineer a larger oxygen buffering capacity with hemoglobin-like molecules might be a more effective strategy to overcome this limitation and increase starch accumulation, as recently suggested [55].

## 6. Lessons from photosynthesis

The control of flux in the Calvin cycle has been reviewed [63,64] and readers are referred to these reviews for the original literature. One surprising fact is that enzymes regarded as limiting, such as Rubisco because of the extremely large $\Delta G$ (−41 kJ/mol), are not limiting at all in normal conditions when photorespiration is low; in this situation half of the Rubisco can be disposed of without much effect on the photosynthetic flux [65]. Conversely, enzymes like transketolase or aldolase, working close to equilibrium, have definitely a high $C^J$ (higher than rubisco) in normal conditions *in vivo* and can attain values of 1 and 0.3, respectively, in high light, high $CO_2$ [41–43].

To change in the way we look at regulation, the reader is encouraged to try a simple thought experiment. Switch off the light shining onto an ideal leaf and imagine what happens to the concentrations of the metabolites in the Calvin cycle, which we can assume as stable during photosynthesis at a steady state. They will undergo transient changes. Intermediates like ribulose bisphosphate in the presence of active rubisco and $CO_2$ will react to give 3PGA. The reaction is highly favoured in standard conditions ($\Delta G^{\circ\prime}$ = −35.1 kJ/mol) as well as *in vivo* ($\Delta G$ = −41 kJ/mol) even after the light is extinguished. ATP production in the dark will be however greatly diminished with an effect on reactions sensitive to ATP concentration. Those far from equilibrium will be less affected by the change in ATP concentration (like phosphoribulokinase, PRK), than those close to equilibrium, like 3PGA kinase (remember that phosphorylation of a carboxylic group by ATP is less favored than phosphorylation of an alcohol). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity will decrease upon lowering of NADPH concentration (expected to be higher in the light) and an increase in $P_i$ coming from ATP hydrolysis. This will decrease the GAP concentration with effects on the rate of the five reactions consuming it: TPI, aldolase (two reactions) and trasketolase (two reactions). Because of the stoichiometry of the first reaction catalyzed by aldolase, FBP concentration will be quite sensitive to the GAP concentration: a twofold change in GAP will translate in a fourfold change in F1,6BP [35]. Extinguishing the light will thus slow down several reactions close to equilibrium simply on thermodynamic grounds due to changes in concentrations first of ATP and NADPH and then of several intermediates. Most of the enzymes catalyzing reactions far from equilibrium will not immediately experience a strong change in driving force, but these are those subjected to either redox regulation by thioredoxin or pH/$Mg^{2+}$ activation (FBPase, sedoheptulose bisphosphatase (SBPase), PRK and Rubisco through Rubisco activase). All the enzymes of the Calvin cycle regulated by thioredoxins or other mechanisms are, except GAPDH, working far from equilibrium.

The thought experiment may supply wrong results about the exact sequence of events or which metabolite concentrations are affected most, but the power of the argument remains untouched. A plethora of regulatory mechanisms affecting photosynthetic enzymes are extensively described in plant biochemistry textbooks: pH, $Mg^{2+}$, reduction by thioredoxins, carbamylation (dependent on rubisco activase), presence of inhibitors, interaction with other proteins (e.g. rubisco activase, CP12 or 14-3-3 [66]), phosphorylation/dephosphorylation ... etc. Many of these regulations are layered one upon the other or influence each other: rubisco carbamylation influences substrate and inhibitor binding; the inhibitor 2-carboxyarabinitol 1-phosphate is removed by the action of a light-activated phosphatase [67], rubisco activase is modulated by thioredoxin and requires ATP [68]; PRK activity depends on a NADPH-dependent dissociation of the PRK/CP12/GAPDH complex [69,70]. The fact that most reactions far from equilibrium are subject to regulation suggests that regulations are important to prevent these reactions to continue converting substrates into products. Otherwise the 3PGA pool will enlarge enormously, engulfing most of the chloroplastic carbon and $P_i$, certainly a dangerous situation. Similar examples are present in primary carbon metabolism with several compounds acting as inhibitors/activators (e.g. trehalose 6-phosphate and F2,6BP, see [71]) and enzymes subject to regulation by 14-3-3 binding in a phosphorylation dependent manner or by thioredoxins [66,61]. F2,6BP is claimed to coordinate "sucrose synthesis with starch formation and photosynthetic activity" [72], implying that this metabolite controls the flux and yet plants with much reduced levels of it show no or very little change in photosynthesis [73–75] and starch levels are not much affected. Indeed the major alterations in these plants are seen at the metabolite level (soluble and phosphorylated sugar). When sucrose synthesis exceeds sucrose removal, by export or degradation, some cytosolic intermediates tend to accumulate (F6P, G6P, TP ... etc.) and others are depleted ($P_i$). A high 3PGA/$P_i$ ratio will eventually switch on starch biosynthesis in the plastid, but an increase in F2,6BP will help to anticipate this switch by restricting the conversion of F1,6BP into F6P, with smaller variation in several metabolites connected to F6P by reactions close to equilibrium (G6P, UDP-G, G1P, TP). The inability to increase F2,6BP experienced by plants altered in fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase retards, but does not block, the onset of starch synthesis [73–75]. Conversely, the excess sucrose is presumably hydrolysed and this is the cause for the surge in free sugars [75,73]. In other words, it is not the increase in F2,6BP driving the partition of photosynthate in favor of starch instead of sucrose [76], but rather a decrease in sucrose export or degradation that causes a increase in F2,6BP. This, on turn, restricts FBPase activity, preventing the build up of excess sugar and sugar phosphates. Once again, metabolic regulation described above is not designed for flux, but rather for concentration control. This is not to say that manipulating mechanisms for concentration control is not going to have any effect on flux: restricting the activity of just one enzyme in a pathway is obviously going to immediately affect flux since the modulation of flux is an asymmetric problem [2], see also Section 1, but the effect will mostly be transient. An increase in the intermediate metabolites upstream of the 'restricted' enzyme and a drop in those downstream augments the driving force, which compensates for most of the reduction in enzyme activity [77,54]. Thus the main effect will be on metabolites and not on flux [3]. The coordinate action of more control mechanisms activating several enzymes in parallel will modulate the flux, but this could not happen if the flux carrying capacity was not already there in the first place.

## 7. Implications for biotechnology

The degree of flux control exerted by an enzyme over a certain flux is quantified by the flux control coefficient ($C^J$). Its value depends not only on the quantity or the properties of the enzyme in question, but also on the quantity or the properties of any other enzyme in the system. In other words, $C^J$ is not a local property, dependent only on the characteristics of the isolated enzyme, but it is a systemic property dependent on the system as a whole [2,3]. $C^J$ values can be experimentally gathered by measuring flux changes upon small reductions in enzyme quantity and they are thus valid

indicators of flux control. One basic idea of Metabolic Control Analysis is that flux control in metabolic pathways is shared among all the enzymes, albeit not necessarily evenly distributed. Since the sum of all $C^J$ in a pathway is equal or close to 1 [2,3,10], it is clear that most $C^J$s must be small. The issue is complicated by the presence of negative $C^J$s (augmenting an enzyme in a branch will decrease flux in another branch, therefore the enzyme $C^J$ on the flux of the other branch will be negative), by the difficulty to define exactly the boundaries of the pathway [78] and also by non-ideal conditions. It is in any case clear that many enzymes have small $C^J$s in wild type conditions [5,6,63,2] and that therefore single enzyme manipulations will bring about small flux increments and a greater change in metabolite distribution [54,3,77] unless the enzyme has a large $C^J$ [2,28].

Which is the best strategy to achieve large flux changes aimed at by metabolic engineering? It depends on the situation. In certain cases the flux might be limited by the flux carrying capacity and therefore the strategy is to increase all or most enzymes in the pathway. In other cases, consumption/removal of a pathway product is the process with the highest $C^J$. Good examples of this are production of ATP or building blocks such as aminoacids or lipids [79–83,57]. In this case flux should increase by increasing consumption (see also Section 8.). In other cases the major limitation is the supply of precursor (e.g. [84]) reminding us that pathway definition is rather arbitrary [78].

The strategy to increase the activity of all or most enzymes in a pathway was proposed as a general method to increase flux [85,2]. There is abundant evidence that flux increases *in vivo* are mediated by a parallel activation (also known as 'multisite modulation') of several enzymes in a pathway (for some examples, see [86–88], see also in [4,54] for additional references). Indeed, a major metabolic pathway like oxidative phosphorylation shows a very large change (>100 times) in flux with little changes in concentration of intermediates of glycolysis and TCA cycle, and this is best interpreted as the result of a parallel activation of enzyme activity [86]. Flux increases, as a result of metabolic engineering of endogenous pathways, are probably more difficult to achieve in primary metabolism (e.g. starch), where enzyme activity can sometimes be at its physical limit or so high as to represent a significant share of total cellular protein [40]. A further increase will possibly affect overall performance, or be limited by energy supply [29]. Conversely, the strategy should be pursued more vigorously, rather than targeting supposed limiting steps, with secondary metabolism, where flux increases are probably easier to achieve. The engineering of new, high flux pathways, such as the biosynthesis of the cyanogenic glucoside dhurrin, can occur with no changes in the amino acid concentrations serving as precursors [89]. This example supports the idea that amino acids fluxes are driven by demand. Strategies based on parallel activation, where applicable, are the best choice for increasing flux because they retain not only metabolite homeostasis, but also flux control distribution. Nice examples of parallel activation at the transcriptional level are those mediated by transcription factors, as exemplified by recent work on the glucosinolate [90–94] flavonoid [95–98] and alkaloid pathways [99].

An interesting strategy would be to target regulatory components (e.g. signal transduction elements, signals or sensors) responsible for the activation of transcription factor regulating metabolic pathways. Interesting and unexplored targets are for instance riboswitches, RNA sensors that affect gene expression via their capacity to bind small molecules. A riboswitch for thiamin pyrophosphate was recently identified in *Arabidopsis* and seems to be widespread in plants [100–102]. Something similar might be present in the *Arabidopsis* biotin pathway [103]. A riboswitch binds to (thereby sensing the level of) the end product of a metabolic pathway, triggering a repression of gene expression. It is easy to envisage that weakening the binding of the sensor to its target should result in an increased steady state level of the end product.

Why is it then that many enzymes catalyzing readily reversible reactions have substantial $C^J$? The secret of the answers lies in the sentence by Mark Twain quoted at the beginning: it is just about the right quantity to perform their function and to avoid the deleterious effect of a null mutation in the heterozygous state: the mutation will have a minor effect on flux and a more pronounced effect on metabolites (more substrates and less products). Why not more enzyme? It would drain too much of resources, which are finite, since many of these enzymes represent a substantial portion of cellular proteins. Why not less? It would further increase the driving force and translate into an appreciable flux reduction.

## 8. Get smarter

One interesting corollary of the concept of parallel activation is that metabolomic approaches will not be informative over physiological changes [3], i.e. it is not sensible trying to relate flux changes with intermediate concentration except for terminal metabolites. Conversely, mutants can reveal a lot about control mechanisms and metabolic pathways (e.g. [104]), because large changes in activity of a single enzyme are expected to cause changes in concentration around the engineered step, rather than changes in flux [3,54,76,53,105,56]. In Metabolic Control Analysis formalism, the amount of control exerted on a metabolite concentration is described by the concentration control coefficient ($C^{S_j}_{E_i}$), where $S_j$ stands for a generalized substrate and $E_i$ a generalized enzyme). It would be greatly beneficial to start measuring these coefficients as a way to describe concentration control quantitatively.

The creation of metabolic shortcuts [54] is a possibility not much exploited. Two recent examples highlight its potential: a chloroplast bypass of photorespiration was engineered through the expression of glycolate dehydrogenase, glyoxylate carboxyligase and tartronic semialdehyde reductase [106] or through glycolate oxidase and malate synthase [107,108]). These caused substantial increases in carbon assimilation and growth rate and reduction in photorespiratory flux.

Improving the amino acid content is achieved more easily by acting on the side of the demand rather than manipulating control mechanisms (e.g. sensitivity to feedback inhibitors) to deregulate the supply [58]. Demand can be increased by sequestration into different compartments [4]. For instance, Wu and Birch [109] were able to double the sugar content of sugarcane, by engineering a sucrose isomerase activity in the vacuole. Sucrose thereby becomes converted into isomaltulose, a more stable isomer ($K'_{eq}$ is around 45, see [110]), which accumulates. This is a remarkable achievement since there has been basically no increase in sugar content of sugarcane over the last 40 years [111]. Another interesting strategy to increase demand is to sequester the product in polymers (e.g. proteins instead of free amino acid). Applying this strategy by expressing genes rich in one or more essential amino acid [112–115] or by altering tRNAs to increase the fraction of codons translated into lysine [116,117] was usually more successful than trying to boost free amino acid levels by tampering with metabolite homeostasis (e.g. [118,62]). For additional references on both strategies, see: [119,120]. Zhu and Galili [62], for instance, demonstrated that free lysine increases 80 times by combining the inactivation of lysine degradation with a desensitized enzyme in *Arabidopsis*. Since free lysine is just a small fraction, total seed lysine increased 'only' 4 fold, still a remarkable achievement. There where however major changes in several amino acid and the highest overexpressors had severe germination problems. When the goal is increasing concentration of a given intermediate metabolite not subject to regulation by demand, then

the (trivial) strategies of choice are the inactivation/reduction of an enzyme consuming the metabolite or the overexpression of an enzyme producing it [54,77].

## 9. Concluding remarks

The prevailing dogma (e.g. [121]), as denounced by Geigenberger et al. [6], was, and still is, that "only the enzymes that catalyze irreversible reactions are relevant for regulation [of flux]". This belief is fundamentally wrong. As I tried to argue here, first of all enzymes catalyzing irreversible reactions have to be regulated in some way to prevent metabolite excesses. Secondly and intimately connected, regulatory mechanisms altering enzyme activity on a short time scale are primarily relevant for maintaining metabolite homeostasis, not for the control of flux. It is also believed that only those enzymes subjected to some regulation (be it allosteric or post-translational modification) could actually regulate the flux. This has also turned out to be wrong, because many unregulated enzymes have substantial flux control. Thus a flux change is mediated by the simultaneous (parallel) activation of most if not all reactions. But to achieve a flux increase, the capacity for flux (a pipe large enough to sustain the required flow) must already be there. When the flux bearing capacity is missing, then there is no way to get the increase. As a corollary, enzymes catalyzing reactions close to equilibrium need to be in large excess in respect to the actual pathway flux, but need not be regulated for maintaining metabolite homeostasis. This is because the reaction regulates its flux automatically.

As clearly stated in a recent review, "the new holistic view of the metabolic network must also include a quantification of the control exerted by each enzyme" [78], surely not an easy task. Confusing control mechanisms employed for different purposes (flux and concentration) will not help solving the puzzle nor improve yields [51]. The almost total disconnection between 'regulated' enzymes and 'flux-controlling' enzymes should have rung the bell a long time ago. Rethinking control means seeing metabolite and flux control as two separate concepts [57,58]. It is clear that I am invoking here a sort of kuhnian paradigm change [122] to occur in biochemistry at large, a paradigm at hand for many years now [1,123,10,57,85,3]. The idea that many forms of 'control' of enzyme activity are relevant to metabolite homeostasis, not flux control, and that metabolite homeostasis is important to avoid the constraints of moiety conservation, may take some time to appear in textbooks and, most importantly, in research labs. This is necessary so that the potential of (plant) biotechnology can be more fully exploited.

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