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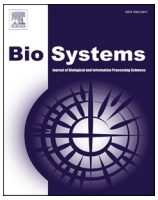

On paradoxes between optimal growth, metabolic control analysis, and flux balance analysis

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ABSTRACT

In Microbiology it is often assumed that growth rate is maximal. This may be taken to suggest that the dependence of the growth rate on every enzyme activity is at the top of an inverse-parabolic function, i.e. that all flux control coefficients should equal zero. This might seem to imply that the sum of these flux control coefficients equals zero. According to the summation law of Metabolic Control Analysis (MCA) the sum of flux control coefficients should equal 1 however. And in Flux Balance Analysis (FBA) catabolism is often limited by a hard bound, causing catabolism to fully control the fluxes, again in apparent contrast with a flux control coefficient of zero. Here we resolve these paradoxes (apparent contradictions) in an analysis that uses the 'Edinburgh pathway', the 'Amsterdam pathway', as well as a generic metabolic network providing the building blocks or Gibbs energy for microbial growth. We review and show that (i) optimization depends on so-called enzyme control coefficients rather than the 'catalytic control coefficients' of MCA's summation law, (ii) when optimization occurs at fixed total protein, the former differ from the latter to the extent that they may all become equal to zero in the optimum state, (iii) in more realistic scenarios of optimization where catalytically inert biomass is compensating or maintenance metabolism is taken into consideration, the optimum enzyme concentrations should *not* be expected to equal those that maximize the specific growth rate, (iv) optimization may be in terms of yield rather than specific growth rate, which resolves the paradox because the sum of catalytic control coefficients on yield equals 0, (v) FBA effectively maximizes growth yield, and for yield the summation law states 0 rather than 1, thereby removing the paradox, (vi) furthermore, FBA then comes more often to a 'hard optimum' defined by a maximum catabolic flux and a catabolic-enzyme control coefficient of 1. The trade-off between maintenance metabolism and growth is highlighted as worthy of further analysis.

1. Introduction

Mammalian cells may be optimal in terms of complex functions such as their contribution to the fitness of the entire multicellular organism (Damiani et al., 2017), the production of fat from glucose (Fell and Small 1986), or the production of neurotransmitters at certain stages in development (Liu and Westerhoff 2023). Microbial cells might be expected to be simpler, and therefore just optimal with respect to their own maximum growth rate, although that optimum may not always have been reached completely (Wiser et al. 2013). Particularly microorganisms with short cell cycles evolve rapidly towards maximal specific growth rate (Groeneveld et al. 2009), probably because enzyme concentrations are readily altered by mutations in the promoter regions of genes. Using flux balance analysis (FBA (Orth et al. 2010)) of the genome-scale metabolic map of the organism, the growth rates of a number of *E. coli* strains were calculated to correspond to those expected for maximal growth rate (Edwards et al. 2001). The implication of the word 'maximal' might seem to be that growth rate should remain the same when any enzyme is tuned up or down, i.e. that all flux control coefficients should equal zero. Such optimality had indeed been observed experimentally for the dependence of balanced growth rate of *E. coli* on the amount of the proton pumping ATPase (Jensen et al. 1993a, 1993b).

However, the condition of being in such a state of maximal growth rate is in apparent contradiction with one of the laws of Metabolic

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Control Analysis (MCA), i.e. that the total control of any steady state flux by all enzymes in the network should be equal to 1 (Kacser and Burns 1973; Heinrich and Rapoport 1974). This should imply that optimality cannot be achieved: it should always be possible to increase the growth rate, because there should always be at least one process with a flux control coefficient higher than zero, which could then be tuned up to enhance growth rate (Kholodenko et al., 2000). The optimality computed by FBA versus this impossibility of optimality apparently implied by MCA, is the paradox that we address and resolve in this paper. Its resolution leads us to additional issues of interest such as the trade-off between maintenance and growth.

2. Results

2.1. Preliminary purely metabolic control analysis of optimization of growth rate

In terms of MCA history, Fig. 1A corresponds to the 'Edinburgh-pathway' (Kacser and Burns 1973). This pathway consists of two reactions linked by a single metabolic intermediate X. The concentrations of the pathway substrate S and product P are fixed and the reaction rates and the concentration of X are variables. In the present context, the pathway corresponds to catabolism of the growth substrate into a set of metabolic intermediates represented by X, followed by anabolism into biomass P (Hellingwerf et al., 1982). Fig. 1B shows the 'Amsterdam pathway', which has a 'leak' (called 'maintenance') in addition to the two processes of the Edinburgh pathway. In a purely metabolic perspective the enzyme levels are fixed in both types of pathway, save explicit modulation.

According to the flux-control summation law (Kacser and Burns 1973; Heinrich and Rapoport 1974; Westerhoff 2023), the sum of the three (two for Fig. 1A) control coefficients with respect to the steady state growth rate (which we here denote by J_g, but see below) should equal 1 both for Fig. 1A and for Fig. 1B:

$$C_1^{J_g} + C_2^{J_g} = C_c^{J_g} + C_a^{J_g} + C_m^{J_g} = 1 \qquad \text{(Eq. 1)}$$

Consequently, it is mathematically impossible for the growth rate to be maximal (and hence optimal) with respect to all three (Fig. 1B) or two (Fig. 1A) enzyme activities simultaneously: For, in such an optimal case and in this purely *metabolic* control analysis all three or two flux control coefficients should equal zero at the same time with the consequence that their sum should equal 0 and not 1.

However, both the growth rate V_g (unit: gram dryweight per second, or mg dryweight per hour) and the biomass concentration B *(unit: gram dryweight)* in the culture should depend on time, the biomass may not be strictly proportional to the total enzyme levels, an increase in enzyme concentration is not the same as an increase in catalytic activity alone, and modulation of one enzyme may cause changes in another enzyme level, due to constraints between them. This suggests we are in need of a more thorough analysis, addressing the control of time dependent properties such as population growth rates and biomass concentrations, and resolving the paradox.

2.2. (Balanced) growth

The (net) population growth rate $V_g(t)$ $\left(\stackrel{\text{def}}{=}\frac{dB(t)}{dt}\right)$ is defined as the increase with time of the biomass $B(t)$. This differs from the *specific* growth rate, which is defined as the population growth rate per unit biomass $\mu(t)$ $\left(\stackrel{\text{def}}{=}\frac{V_g(t)}{B(t)}\right)$. For microorganisms growing in a chemostat at steady state, the (net) population growth rate V_g may be measured by multiplying the dilution rate of the chemostat (which equals the *specific* growth rate μ of the cells) by the concentration of the cells ((Snoep et al., 1994), see also below). In batch culture, both the growth rate and the substrate consumption flux will vary with time, but intracellular metabolism is faster than this (in the sense of having shorter inherent relaxation times due to the small intracellular volume as compared to the extracellular volume) and can thereby be assumed to be at an intracellular (quasi (Heinrich et al. 1977)) steady state. As the substrate concentration is often far above the Michaelis-Menten constant of the transporter, that concentration is sensed as constant by intracellular metabolism, so that the intracellular state also becomes independent of time as do the probabilities of cell division and cell death: the death rate constant μ_{death}, the division rate constant $\mu_{division}$, and the mass (b) of an individual cell are then independent of time; the system is one of 'balanced growth' (Stouthamer and Bettenhaussen 1975). μ_{death}, $\mu_{division}$, and $\boldsymbol{b}$ are functions of the metabolic capabilities of the cells and can be subject to experimental or evolutionary moderation.

Considering the cells to be asynchronous with respect to their cell cycle and in balanced growth from before t = 0, integration of the corresponding differential equation for cell number N(t) predicts an exponential increase (or decrease) of both the biomass ($B(t) = b{\cdot}N(t)$), *i.e.* the amount of biomass at time *t*, in gram dryweight or C-moles) and the 'population growth rate' $\left(V_g(t)\stackrel{\text{def}}{=}\frac{dB(t)}{dt}\right)$ with time t, whereas the *specific* growth rate $\frac{V_g(t)}{B(t)} = \mu$, the cell growth rate $v_g = V_g/N$ and the biomass

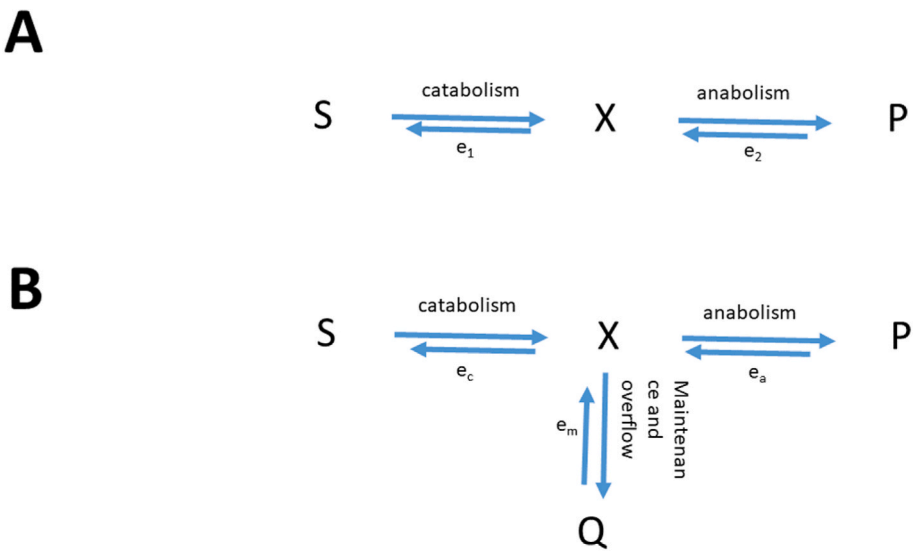

Fig. 1. Simplified pathway representations of microbial growth. A: the quasi 'Edinburgh pathway' (Kacser and Burns 1973). X is a variable metabolite concentration, whilst S and P are present at fixed concentration (or at concentrations far above the corresponding Michaelis constants). e_1 and e_2 catalyze the reactions from S to X and from X to P, respectively, which may represent catabolism and anabolism-growth. B: the 'Amsterdam pathway' (Westerhoff and Van Dam 1987) with fixed concentrations of substrate S and of its two products P and Q. The reaction from X to P represents anabolism = biomass synthesis and that from S to X catabolism. The reaction from X to Q represents maintenance and overflow metabolism 'm'. Enzyme (consortia) e_c, e_a and e_m catalyze the reactions from S to X (catabolism), from X to P = biomass (anabolism or growth), and from X to Q (maintenance), respectively.

per cell B/N = b are all independent of time:

$$\frac{V_g(t)}{V_g(0)} = \frac{B(t)}{B(0)} = \frac{N(t)}{N(0)} = e^{\mu \cdot t} \quad \text{(Eq. 2)}$$

Here we note that in balanced growth the specific growth rate equals the difference between the growth rate constant and the death rate constant:

$$\mu \stackrel{\text{def}}{=} \frac{V_g(t)}{B(t)} = \mu_{division} - \mu_{death} \quad \text{(Eq. 3)}$$

This equation shows that over longer times both the biomass concentration in the cell culture and the population growth rate increase enormously and continue to do so. Formally no steady state is attained therefore and the summation laws derived for steady state will not apply to biomass levels and the population growth rates under these conditions (they may apply in stationary phase). (Kacser and Burns 1973) solved this problem by focusing on what one might call a system of 'mother cells' that create daughter cells that leave the system. This led to a description of intracellular metabolism and specific growth (see below) with added fluxes of metabolites and biomass 'to expansion'. The summation laws then applied again. We will here follow a different route, and continue to describe the entire cell culture (or mammal) as it develops in time.

We shall use the time coefficient defined for any variable z by (Acerenza et al. 1989; Westerhoff, 2023):

$$C_t^z \stackrel{\text{def}}{=} \left(\frac{\partial \ln|z|}{\partial t}\right)_{dk_l=0 \text{ for all } l} \quad \text{(Eq. 4)}$$

$|z|$ refers to the absolute magnitude of the property; k_l to any of the complete set of rate constants with dimension inverse time. Below we shall abstain from being explicit about the absolute value; any logarithm will be defined as the logarithm of the absolute value of its argument. ln refers to the natural (e-based) logarithm; and to partial differential with the conditions of the partial differentiation being indicated by the subscript. For 'balanced growth' one finds:

$$C_t^{V_g} = C_t^B = \mu \cdot t. \quad \text{(Eq. 5)}$$

2.3. Generalization of the summation laws to time dependent properties

The definition of these time coefficients makes it possible to generalize MCA to time-dependent systems such as a growing cell culture or organism. For this we also need to define the time-dependent control coefficient (Acerenza et al. 1989; Westerhoff 2008):

$$C_{\_}(k\_j)(z(\hat{t})) \stackrel{\text{def}}{=} ((\partial ln|z(t)|)/(\partial \ln(k\_j)))_{\_}(dk\_l = dt = 0 \text{ for all } l \neq j) \quad \text{(Eq. 6)}$$

This is a generalization of the control coefficient defined for steady state flux by (Heinrich and Rapoport 1973; Kacser and Burns 1973; Burns et al., 1985), both to more properties and to properties that are time dependent. Time dependent control coefficients like $C_{k_j}^{B(t)}$ and $C_{k_j}^{V_g(t)}$ are important here because we wish to discuss the control of the (non-specific) growth rate and the biomass concentration in a culture, whilst these properties are varying with time as described above.

In order to avoid confusion around time dependent control coefficients, we point out that there are three types of these. The ones used here and in (Acerenza et al. 1989) address properties that themselves vary appreciably with time. They ask to what extent a persistent variation of any time-independent catalytic parameter k_j causes a change in such a variable at time t. The ones used by (Heinrich and Reder 1991; Westerhoff and Chen 1984) again address the extent to which a persistent variation in a catalytic parameter changes that variable at time t but now for the cases where that variable is effectively at steady state but has been subject to an infinitesimal perturbation and is relaxing back to the steady state. And then there are control coefficients with respect to integral characteristics of dynamic properties, such as frequencies of oscillations (Bier et al., 1996; Kholodenko et al. 1997), times of half maximal increase or decrease of protein phosphorylation in signal transduction (Hornberg et al., 2005a, 2005b), enzyme characteristic times (Heinrich et al. 1977), and pathway-transit times (Torres and Meléndez-Hevia 1992; Kholodenko et al., 1996). The magnitude of the latter four control coefficients need not vary with time.

We shall consider properties of living organisms to be functions of the specific catalytic activities within them (Westerhoff and Van Dam 1987). For ideal biochemical pathways (i.e. no channeling and every chemical and transport reaction catalyzed by a single enzyme or transporter (Kholodenko et al., 1995)), we shall use k_j to refer to the forward maximal catalytic activity of enzyme or transporter e_j, expressed in unit product concentration (or mole number) per time unit per amount of enzyme. At given concentrations of all the metabolites and well-defined values of the corresponding Michaelis-Menten and activation/inhibition constants, and of the equilibrium constant (Goldberg et al., 2023)), the rate V_j of any reaction is proportional to this catalytic activity, i.e.:

$$V_j = E_j \cdot k_j \cdot \left(1 - e^{\frac{\Delta_j G}{RT}}\right) \cdot \varphi_j \left(\frac{[x_{jl}]}{K_{M.X_{jl}}}\right) \quad \text{(Eq. 7)}$$

V_j represents the total rate of the reaction j in the reaction vessel (in kat = Moles/second or mMol.minute). E_j represents the total amount of catalyst j (i.e. enzyme or transporter, or virtual substitute in the rare cases that the reaction is not catalyzed by either of these; in Moles or microgram) in that vessel. $k_j = k_{cat+,j}$ (Cornish-Bowden 2012) is the catalytic rate constant of that enzyme (unit: /second or mole/microgram/second). $e^{\frac{\Delta_j G}{RT}}$ is the value of the exponential function with as argument the Gibbs energy of reaction normalized by the gas constant multiplied by the absolute temperature. This Gibbs energy is a function of the metabolite concentrations $[x_{jl}]$ and the equilibrium constant. φ_j is a (dimensionless) function that represents the catalyst kinetics (Hill 1977; Cornish-Bowden 2012). It corresponds to the extent of saturation of the enzyme with its substrates (Westerhoff and Van Dam 1987). $[x_{jl}]$ is the intracellular concentration (in Molar) of the l^{th} intracellular metabolite that affects reaction *j; in Eq. 7 it is divided by the corresponding Michaelis constant also with Molarity as dimension*. In balanced growth they are also time independent, which means that their differential equation equals zero:

$$0 = \frac{d[x_{jl}]}{dt} = \sum_k \nu_{jlk} \cdot \frac{V_k}{N \cdot v_1} - (\mu_{division} + \mu_{death}) \cdot [x_{jl}], \quad \text{(Eq. 8)}$$

where the final terms correspond to the fluxes to expansion and cell death, v_1 to the volume of 1 cell, and ν_{jlk} the stoichiometry at which metabolite jl occurs in reaction k. Through these equations the intracellular concentrations and thereby the reaction rates per cell as well as the division and death rate constants are functions of the enzyme concentrations in the cells $e_k \stackrel{\text{def}}{=} \frac{E_k}{N \cdot v_1}$, the catalytic rate constants (k_j), the Michaelis-type constants $K_{M.X_{jl}}$ and the reaction stoichiometries. Traditionally, the stoichiometries are considered immutable (see however the recently proposed gear shifting (Zhang and Westerhoff 2023), but we will not break with this tradition here). We will do the same for the Michaelis type constants (see however (Kholodenko et al., 1998)). We will examine how concentrations and fluxes depend on the catalytic rate constants and the enzyme concentrations within the cells. The set of k_j's should be complete in that it contains all parameters with time dimensionality −1 (Westerhoff 2023). The arithmetic product of the catalytic rate constant k_j and the enzyme concentration E_j, *i.e.* the maximum rate of $V_{max+,j}$ (cf. Eq. 34) also has time dimensionality minus 1.

The flux-control summation law of MCA is a special case of a generic property of deterministic non-equilibrium systems: their states are independent of the units in which time is expressed (Westerhoff 2023). For instance, the system should be the same at $t = 1$ min as at $t' = 60$ s or at $t'' = 120$ half-seconds. More generally ($\lambda = 60$ and 120 in the examples):

$$t' = \lambda \cdot t, \quad \text{(Eq. 9)}$$

where t' and t only differ in terms of the time units they are expressed in. (This property can be understood from the above differential equation before its time dependence has disappeared.) As a consequence of this unit independence:

$$k_j' =_{every\ j} \frac{k_j}{\lambda} \quad \text{(Eq. 10)}$$

For properties $v(t)$ (like the population growth rate V_g) with time dimensionality −1, this leads to:

$$v\left(\left(k_j'\right)_{for\ all\ j}, t'\right) = \lambda \bullet v\left(\left(\frac{k_j}{\lambda}\right)_{for\ all\ j}, \lambda \bullet t\right) = v\left((k)_{for\ all\ j}, t\right)\ for\ all\ \lambda \quad \text{(Eq. 11)}$$

So that the derivative with respect to should equal zero:

$$0 = \sum_{all\ j} \frac{\partial ln\left(v\left((k_j)_{for\ all\ j}, t\right)\right)}{\partial \ln(\lambda)} = \frac{\partial ln\left(\lambda \cdot v\left(\left(\frac{k_j}{\lambda}\right)_{for\ all\ j}, \lambda \cdot t\right)\right)}{\partial \ln(\lambda)} =_{\lambda=1} 1 + C_t^{v(t)} - \sum_{all\ j} C_j^{v(t)}. \quad \text{(Eq. 12)}$$

Consequently, the sum of all the v control coefficients equals 1 plus the time coefficient of v (Acerenza et al. 1989; Westerhoff, 2023):

$$\sum_{all\ j} C_{k_j}^{v(t)} = 1 + C_t^{v(t)} \quad \text{(Eq. 13)}$$

In the control coefficients the time dimensions drop out because they are ln/ln derivatives (Eqs. 4 and 6; $d\ln|z| = dz/z$, whereby the dimension drops out), and 1 is without dimensions accordingly. For steady states, where $C_t^{v(t)} = 0$, this becomes the flux control summation law of MCA (Kacser and Burns 1973; Heinrich and Rapoport 1974). For balanced growth this reads for the population growth rate V_g:

$$\sum_{all\ j} C_{k_j}^{V_g(t)} = 1 + \mu \cdot t \quad \text{(Eq. 14)}$$

In order to derive the time dependent summation law for concentration and yield control coefficients, we address $X(k_j, t)$, which represents any system variable that has a dimension independent of time, such as concentration, growth yield in terms of flux ratio, Gibbs energy of reaction, and thermodynamic efficiency (see below). Requiring that any property of this 'concentration type' should be independent of the time unit used, hence independent of the factor λ (certainly in the limit to a very small change in time unit; hence we consider the limit to $\lambda = 1$), we find (Westerhoff 2023):

$$0 = \sum_{all\ j} \frac{\partial ln\left(X\left(\left(\frac{k_j}{\lambda}, t\right)_{for\ all\ j}, \lambda \cdot t\right)\right)}{\partial \ln(\lambda)} =_{\lambda=1} C_t^{X(t)} - \sum_{all\ j} C_{k_j}^{X(t)}, \quad \text{(Eq. 15)}$$

and hence the generalization of the summation law for concentration control (Westerhoff 2023):

$$\sum_{all\ j} C_{k_j}^{X(t)} = C_t^{X(t)}. \quad \text{(Eq. 16)}$$

For steady states (i.e., $C_t^{x(t)} = 0$) the above equation becomes MCA's summation law for steady-state concentrations (Kacser and Burns 1973; Heinrich and Rapoport 1974). The generalized version of this law is also valid for time dependent states such as transients in signal transduction (Hornberg et al., 2005a, 2005b). It reflects that when a concentration decreases with time, the sum of all its concentration control coefficients should be negative; proportional activation of all processes at the same time should accelerate the decrease proportionally. It also implies that the pathways that make ATP, collectively exercise less control on the decay part of the transient ATP level following a substrate pulse, than the enzymes that consume the ATP.

In the case of balanced growth, which is outside steady state, one finds for the total biomass concentration:

$$\sum_{all\ j} C_{k_j}^{B(t)} = \mu \cdot t, \quad \text{(Eq. 17)}$$

Showing that after a long time of growth the control exercised by an intracellular process on the amount of biomass obtained, can be much larger than 1. A persistent 10% activation of an intracellular process may increase the yield of the culture manyfold.

2.4. 'Smooth' and 'hard' optimization

We shall consider the maximization of a function $f(k_j, e_j, t) > 0$ with respect to variation in an enzyme concentration e_j. Where the function is maximal the enzyme concentration will be called optimal. In our 'smooth-optimum' interpretation we assume that the enzyme concentration can assume values both above and below this optimal concentration, and that the value of the function is smaller than maximal in both cases. The condition for this type of 'smooth' optimality is thereby that the function is locally at the top of an inverse parabola. In linear terms this condition requires that:

$$\frac{df(e_j, e_l)}{de_j} = 0\ and\ \frac{d^2 f(e_j, e_l)}{(de_j)^2} < 0 \quad \text{(Eq. 18)}$$

In MCA one is used to logarithmic terms. The equivalent condition is then:

$$\frac{dln(f(e_j, e_l))}{dln(e_j)} = 0\ and\ \frac{d\frac{dln(f(e_j, e_l))}{dln(e_j)}}{de_j} < 0 \quad \text{(Eq. 19)}$$

An alternative to this 'smooth optimum' is a 'hard optimum', which obtains if:

$$\left(\frac{\partial f(e_j, e_l)}{\partial e_j}\right)_{de_l=0\ for\ all\ l \neq j} > 0\ and\ de_j = 0\ for\ e_j \geq e_{jmax} \quad \text{(Eq. 20)}$$

i.e. e_j itself is maximal due to some constraint. The subscripted $de_l = 0$ signifies that, as the activity of enzyme j is modulated $de_j \neq 0$, the activities of all other enzymes (e_l for any value of l not equal to j) are kept constant.

2.5. Specification of the paradox between growth rate being maximal and the generalized summation law of MCA

In the above equations total derivatives are used, not partial derivatives. $\frac{dln(f(e_j, e_l))}{dln(e_j)}$ is a so-called enzyme control coefficient, which can differ from the control coefficient proper (the 'catalytic control coefficient', which is the corresponding partial derivative with respect to the catalytic rate constant; see below and (Kholodenko et al., 1995; Schuster and Heinrich 1992)). Although we assume that enzyme j is the only enzyme that is *directly* modulated, the other enzymes may also change as a consequence of the change in e_j if the enzyme concentrations depend on each other due to some constraint (Heinrich et al. 1987; Klipp and Heinrich 1999) or by cross-influence through regulated gene expression (e.g. (Snoep et al., 2002)). If the other enzyme concentrations remain constant, the total derivatives become partial derivatives and

correspond to the catalytic control coefficients, so that in the optimal state the requirement is:

$$C_{k_j}^{f} = \left(\frac{\partial ln\left(f\left(e_j, e_l\right)\right)}{\partial ln\left(k_j\right)}\right)_{dk_l=0 \text{ for all } l\neq j \text{ and } de_l=0 \text{ for all } l} =_{no\ constraints} \left(\frac{\partial ln\left(f\left(e_j, e_l\right)\right)}{\partial ln\left(e_j\right)}\right)_{de_l=0 \text{ for all } l\neq j \text{ and } dk=0 \text{ for any } l} = 0 \quad \text{(Eq. 21)}$$

Considering f to represent the (population) growth rate and requiring this growth rate to be optimal with respect to independent variation of all enzyme concentrations (if these are indeed independent) then leads to the apparent contradiction (as indicated by the = ? signs):

$$0 =? \sum_{allenzymes} \left(\frac{\partial ln\left(V_g\left(e_j, e_l\right)\right)}{\partial ln\left(e_j\right)}\right)_{de_l=0 for all l\neq j and dk=0 for any l} =? \sum_{allenzymes} C_{k_j}^{V_g} =? 1+\mu\cdot t \gg 1 \quad \text{(Eq.22)}$$

The right hand side of the equation is based on the assumption of balanced growth (see above). As we expect that growth *can* be smooth-optimal with respect to the enzyme concentrations and the summation law has been proven mathematically, we conclude that the equation constitutes a paradox (we use the word paradox in the meaning of an apparent contradiction that is not really a contradiction). Apparently then, the resolution of the paradox noted here should reside in (i) the property being optimized to a smooth maximum not equaling the total population growth rate V_g, but rather being a property for which the control coefficients do sum up to 0, (ii) the enzyme concentrations being dependent properties due to a constraint between them, or (iii) the optimality requested being a hard rather than a smooth optimum. We shall now discuss these possibilities.

2.6. Optimization target: population growth rate, specific growth rate, growth yield, or growth efficiency?

Prokaryotes have existed for 4 billion years on this planet and some have cell cycle times below the hour. The trillions of cell cycles, the fact that expression levels are changed readily by mutations in the genes' promotor areas, and the results of long-time cultivation experiments suggest that microbial growth may be optimal or close to (Wiser et al. 2013) optimal. We shall first discuss a scenario in which one should expect microbes to optimize for specific growth rate μ. Subsequently we shall discuss optimization for yield or thermodynamic efficiency.

In the evolution scenario in which repeatedly a small sample of medium escapes to a new location with excessive amounts of growth substrate, before the original culture is wiped out at time t, one finds for the probability for survival of the species in the new habitat:

$$Species\ survival\ probability \stackrel{def}{=} P = \frac{Volume_{sample}}{Volume\cdot b}\cdot V_g(0)\cdot e^{\mu\cdot t} \quad \text{(Eq. 23)}$$

b represents the amount of biomass in one cell and $V_g(0)$ the population growth rate at time zero. For balanced growth the control of this probability by the catalytic rate constant k_j is given by:

$$C_{k_j}^{P} \stackrel{def}{=} \frac{\partial \ln\left(P\right)}{\partial \ln\left(k_j\right)} = C_{k_j}^{V_g(0)} + \frac{\partial \ln\left(P\right)}{\partial \ln\left(\mu\right)}\cdot C_{k_j}^{\mu} = C_{k_j}^{V_g(0)} + C_{k_j}^{\mu}\cdot\mu\cdot t =_{large\ t} \mu\cdot C_{k_j}^{\mu}\cdot t \quad \text{(Eq. 24)}$$

This shows that after many cell cycles under balanced growth conditions, the control on population growth rate at time zero, $C_{k_j}^{V_g(0)}$, can be neglected relative to the control on the specific growth rate. This corresponds to the survival advantage that rapid cell cycling with consequent smaller biomass per individual offers (see however (Marshall et al., 2022)). It shows why some micro-organisms may have optimized towards maximal specific growth rate μ (assuming that maintenance metabolism is not affected during the optimization; see below).

Organisms may not always have lived under the balanced growth conditions that we here considered. Feast and famine may have been more frequent, in which case optimization may have been in terms of growth yield rather than growth rate, or the thermodynamic efficiency of growth may have become important (Westerhoff et al. 1983), as we shall now rationalize: In such a different scenario, a limited amount of growth substrate is available to the microbes, which becomes depleted before the dispersion event. The final cell number n then equals (with s signifying the limiting number of moles of substrate):

$$N(\infty) = y\cdot\frac{s}{b} \quad \text{(Eq. 25)}$$

with s the amount of growth substrate and y the growth yield (in mass of cells per mass of growth substrate), and b again the mass of one cell. If shortly after finishing the growth substrate this culture is dispersed so that every one of its cells ends up in an environment with again a limited amount of growth substrate, then upon multiple repetitions of this procedure, optimization should be towards growth yield $y \stackrel{def}{=} \frac{V_g}{v_c}$ (Bachmann et al., 2016). If however, the cells need to persist for long after substrate depletion, they will begin to die, thereby releasing their own biomass as substrate for growth of the survivors. Then high growth yield and low maintenance metabolism will be important. In yet another scenario, the growth may be limited by the Gibbs energy in the substrates compared to the products of catabolism (ΔG_c). Then the Gibbs energy stored (g_{stored}; dimension Gibbs energy per mole of catabolic substrate) in the new cells (and thereby the thermodynamic efficiency of growth) will matter, which equals:

$$g_{stored} = s\cdot - \Delta G_c\cdot\eta \quad \text{(Eq. 26)}$$

Should the organism be able to choose ('gear shift' (Zhang and Westerhoff 2023)) between various pathways for the same overall catabolic conversion, or between various catabolic Gibbs energies $-\Delta G_c$ (as in overflow metabolism, e.g. (Van Hoek, Van Dijken, and Pronk 1998)), the optimization may be for ΔG_a (ΔG_a is defined as the Gibbs energy in biomass minus the Gibbs energy in the substrates for growth (Westerhoff and Van Dam 1987)). Some organisms thrive on producing certain catabolites such as alcohol, possibly to ward off competing organisms. Their growth may optimize for yield of the corresponding catabolite (Schuster et al. 2008).

For now we shall focus on specific growth rate μ and growth yield y as possible optimization targets and ask whether optimization of either of these properties is consistent with MCA.

2.7. Paradox persists when considering the specific growth rate

The specific growth rate is related to the population growth rate and the biomass through:

$$V_g = \frac{dB}{dt} = \mu\cdot B \quad \text{(Eq. 27)}$$

and can thereby be identified as the 'specific growth rate, i.e. the growth rate per unit biomass.':

$$\mu = \frac{V_g}{B} = \frac{v_g}{b}, \quad \text{(Eq. 28)}$$

where v_g and b are the biomass synthesis rate per cell and the biomass per cell, respectively. The control coefficients with respect to this specific growth rate are then equal to (for balanced growth):

$$C^{\mu}_{k_j} = C^{V_g}_{k_j} - C^{B}_{k_j} = C^{v_g}_{k_j} - C^{b}_{k_j} \quad \text{(Eq. 29)}$$

Specific growth rate μ and biomass per cell b have time dimensionality of −1 and 0 respectively, so that their summation laws read:

$$\sum_{all\ j's} C^{\mu}_{k_j} = 1 + C^{\mu}_{t} \quad \text{(Eq. 30)}$$

$$\sum_{all\ j's} C^{b}_{k_j} = C^{b}_{t} \quad \text{(Eq. 31)}$$

For the same reason Eq. 30 is valid for the cellular growth rate v_g. The time coefficients of the specific growth rate and biomass per cell are both equal to zero in balanced growth, so that:

$$C^{\mu}_{t} = C^{V_g}_{t} - C^{B}_{t} = C^{v_g}_{t} - C^{b}_{t} = 0 - 0 = 0 \quad \text{(Eq. 32)}$$

Consequently, during balanced growth, the summation laws for both the growth rate per cell (v_g) and the specific growth rate amount to 1:

$$\sum_{all\ j's} C^{\mu}_{k_j} = {}_{balanced\ growth} = \sum_{all\ j's} C^{v_g}_{k_j} = {}_{balanced\ growth} = 1 \quad \text{(Eq. 33)}$$

This suggests that making the specific growth rate the target of optimization does not resolve the paradox: the sum of its catalytic control coefficients still equals 1, so that its enzyme control coefficients cannot all be equal to zero in the absence of constraints on the enzyme levels. The same applies to the enzyme control coefficients with respect to cellular growth rate.

2.8. Can the dependence of specific growth rate on enzyme concentrations evade the summation law?

2.8.1. In the absence of constraints and compensation there can be no smooth optimum in the dependence of specific growth rate on enzyme concentrations

In the preceding paragraph, we used the catalytic control coefficients. However, as shown in section 2.5 the enzyme control coefficients are the ones that are directly relevant for optimization. In section 2.3 we showed that in balanced growth the metabolite concentrations and reaction rates depend on the catalytic rate constants and the enzyme concentrations, the arithmetic product of which constitute the corresponding V_{max}:

$$v_{max.j} = k_j \cdot e_j. \quad \text{(Eq. 34)}$$

The same is true for all the state functions of the cells. Some state functions also depend more directly on the enzyme concentrations, however. The amount of biomass per cell for instance may change when a particular enzyme j is activated (e.g. an enzyme controlling lipid synthesis) but will certainly (unless there is compensation, see below) change when the activation is effected by an up-modulation of the enzyme concentration, which directly increases the amount of biomass, since:

$$b = b_0\left(V_{max.j}(e_j), V_{max.l}(e_l)\right) + e_j + \sum_{l\neq j} e_l, \quad \text{(Eq. 35)}$$

where b_0 represents the catalytically inert biomass, the concentration of which may or may not change (increase if b_0 is coupled to the cells changing mass; decrease if b_0 compensates) when enzyme j is increased. The dependence of the amount of biomass per cell on the concentration of enzyme j, may then be written as:

$$\frac{dln(b)}{dln(e_j)} = \frac{e_j}{b}\cdot\left(1 - \gamma_0 - \sum_{l\neq j}\gamma_l\right), \quad \text{(Eq. 36)}$$

where the gamma's refer to the compensation by a change in the amount of inert biomass and by the amount of the other enzymes, respectively:

$$\gamma_0 \stackrel{\text{def}}{=} -\frac{db_0}{de_j} \quad \text{(Eq. 37)}$$

$$\gamma_l \stackrel{\text{def}}{=} -\frac{de_l}{de_j} \quad \text{(Eq. 38)}$$

If there is no such compensation:

$$\frac{dln(b)}{dln(e_j)} = \frac{e_j}{b}\stackrel{\text{def}}{=}\varphi_j, \quad \text{(Eq. 39)}$$

where φ_j is the fraction of biomass that is enzyme j. For the growth rate per cell, in general:

$$v_g = v_g\left(V_{max.j}(e_j), e_j, V_{max.l}(e_l), e_l\right) \quad \text{(Eq. 40)}$$

Our assumption of ideal metabolic pathways, implies that the growth rate only depends on the level of enzyme j through $V_{max.j}(e_j)$ and (if there is regulation of gene expression) $V_{max.l}(e_l)$ so that:

$$\left(\frac{\partial ln(v_g)}{\partial ln(e_j)}\right)_{dV_{max.j}=0} \quad \text{(Eq. 41)}$$

and

$$\frac{dln(v_g)}{dln(e_j)} = C^{v_g}_{k_j} + \sum_{l\neq j} C^{v_g}_{k_l}\cdot\frac{dln(e_l)}{dln(e_j)} = C^{v_g}_{k_j} + \sum_{l\neq j} C^{v_g}_{k_l}\cdot\frac{e_j}{e_l}\cdot\frac{de_l}{de_j} = C^{v_g}_{k_j} - \sum_{l\neq j} C^{v_g}_{k_l}\cdot\frac{e_j}{e_l}\cdot\gamma_l \quad \text{(Eq. 42)}$$

For the specific growth rate:

$$\frac{dln(\mu)}{dln(e_j)} = \frac{dln(v_g)}{dln(e_j)} - \frac{dln(b)}{dln(e_j)} = C^{v_g}_{k_j} - \sum_{l\neq j} C^{v_g}_{k_l}\cdot\frac{e_j}{e_l}\cdot\gamma_l - \frac{e_j}{b}\cdot\left(1 - \gamma_0 - \sum_{l\neq j}\gamma_l\right), \quad \text{(Eq. 43)}$$

For the summation over all enzymes j, this leads to:

$$\sum_j \frac{dln(\mu)}{dln(e_j)} = 1 - \sum_j\sum_{l\neq j} C^{v_g}_{k_l}\cdot\frac{e_j}{e_l}\cdot\gamma_l - \varphi_e\cdot(1 - \gamma_0 - \gamma_e), \quad \text{(Eq. 44)}$$

where γ_e is the compensation by all enzymes except of course e_j (because e_j is set by the modulation and we have assumed that the compensation by the other enzymes is independent of the enzyme that was upmodulated).

If there is no compensation, so that the amount of biomass just increases with the amount of the enzyme that is modulated implying that both γ's equal zero, then the sum of the enzyme control coefficients on the specific growth rate equals the fraction of biomass that is not enzyme:

$$\sum_j \frac{dln(\mu)}{dln(e_j)} = 1 - \varphi_{enzymes} \quad \text{(Eq. 45)}$$

$$\varphi_{inert} \stackrel{\text{def}}{=} 1 - \varphi_{enzymes} \stackrel{\text{def}}{=} 1 - \frac{\sum_j e_j}{b} = \frac{b_0}{b}. \quad \text{(Eq. 46)}$$

φ_{inert} is the fraction of biomass that neither consists of enzymes nor of components that vary proportionally with the enzymes.

If there is no such inert fraction, the sum of the enzyme control coefficients on the specific growth rate equals zero. This answers the interesting thought that is inspired by the flux control summation law: if one were to increase all catalytic activities in proportion then the growth rate per cell should increase in the same proportion. This shows that one can always increase the growth rate at some point in time in what has been called the 'universal method' (Acerenza et al. 1989). The catch however is that the increase in enzyme concentrations will also increase

the biomass per cell, so that the specific growth rate does not increase much unless the fraction of non-enzyme biomass is still substantial.

In practice approximately 55% of cell dryweight is protein (Stouthamer 1973; Valgepea et al., 2013) and assuming that the other biomass components are indeed catalytically inert and invariant, the actual situation is one in which the inert fraction differs substantially from zero. This reduces the sum of the enzyme control coefficients from 1 to 0.45 but not all the way to zero. This has the implication that there cannot be a smooth optimum with respect to all enzyme concentrations; if there is no compensation, the specific growth rate cannot be optimal with respect to the enzyme concentrations.

2.8.2. Yes: When the other enzymes compensate to maintain biomass constant, there is a smooth optimum in the dependence of specific growth rate on enzyme concentrations

2.8.2.1. Arbitrary pathway at constant total enzyme and biomass concentrations. An inherently simpler situation obtains when the compensation is complete in the sense that the total biomass concentration (amount per cell) is constant. Then:

$$\frac{dln(b)}{dln(e_j)}=0 \qquad \text{(Eq. 47)}$$

$$\frac{dln(\mu)}{dln(e_j)}=\frac{dln(v_g)}{dln(e_j)}-\frac{dln(b)}{dln(e_j)}=C_{kj}^{v_g}-\sum_{l\neq j}C_{kl}^{v_g}\cdot\frac{e_j}{e_l}\cdot\gamma_l, \qquad \text{(Eq. 48)}$$

A more specific approach assumes that total enzyme protein should be constant, for instance because enzyme synthesis rate is limited by the ribosome concentration, so that up-modulation of one enzyme should automatically decrease the levels of all the other enzymes and possibly the growth rate; this effect has been called the protein burden effect in bioengineering (Snoep et al., 1995). Then all compensation occurs by the proteins:

$$\gamma_e=\sum_{l\neq j}\gamma_l=1 \qquad \text{(Eq. 49)}$$

Optimality for all enzymes then requires that for any j:

$$\frac{C_{kj}^{v_g}}{e_j}=\sum_{l\neq j}\frac{C_{kl}^{v_g}}{e_l}\cdot\gamma_l \qquad \text{(Eq. 50)}$$

One solution is:

$$\frac{C_{kj}^{v_g}}{e_j}=\alpha_1 \qquad \text{(Eq. 51)}$$

i.e. the ratio of flux control coefficient to enzyme concentration is the same for all enzymes. Applying the summation law for flux control coefficients (Eq. 33) shows that α_1 is equal to the inverse of the total enzyme concentration. For a network of *n* enzymes, Eqs. 47 and 48 constitute *2n* equations in *2n* unknowns (i.e., the *n* enzyme concentrations and the *n* flux control coefficients), which means that Eq. 51 is the only solution for which the dependence of the specific growth rate on all enzymes equals zero. At any rate this analysis shows that the specific growth rate can be optimal with respect to all enzyme concentrations in the pathway (but see below), whilst the summation law of MCA remains valid. Apparently one resolution of the paradox is that the specific growth rate can be optimal provided that the total enzyme concentration is constant.

This conclusion had already been reached for the metabolic flux through linear pathways by (Klipp and Heinrich 1999) but here it is achieved explicitly for the *specific* growth rate. Further extending their Lagrangian undetermined multiplier approach, we here examine the condition in which any of the enzyme concentrations is varied, the total biomass concentration is constant, the catalytically inert biomass (b_0) is linearly related (in this section we consider that it increases with increasing total protein, *i.e.* as the cell gets bigger) to the total enzyme concentration ($b_0 = b_{00} + \beta_b \cdot e_{total}$, with $b_{00}>0$), and the specific growth rate is maximal with respect to that variation. Hereto we require:

$$\frac{\partial(b+\alpha\cdot\mu)}{\partial e_j}=\frac{\partial\left((1+\beta_b)\cdot e_{total}+\alpha\cdot\mu\right)}{\partial e_j}=0 \qquad \text{(Eq. 52)}$$

α is an undetermined multiplier, meaning that the equation should be correct for any of its values, with the effect that both requirements (i.e. constant total biomass and constant specific growth rate) are met. Elaborating the derivative leads to:

$$C_j^{\mu}=-\frac{e_j\cdot(1+\beta_b)}{\alpha\cdot\mu} \qquad \text{(Eq. 53)}$$

Using the summation law for the specific growth rate, one can solve for α and obtain:

$$\left(\frac{e_j}{e_{total}}\right)_{optimal}=C_j^{\mu} \qquad \text{(Eq. 54)}$$

The result is independent of whether the inert biomass does or does not ($\beta_b = 0$) vary with total enzyme concentration and identical to that obtained above and by (Klipp and Heinrich 1999), even though these authors considered the growth rate rather than the specific growth rate (the reason is that in the procedure total enzyme and hence biomass is invariant). With respect to the catalytic control on the specific growth rate one finds again the same value:

$$C_j^{\mu}=C_j^{v_g}-\frac{dln(e_{total}+b_0)}{dln(e_j)}=C_j^{v_g} \qquad \text{(Eq. 55)}$$

Because of the interdependence of the enzyme concentrations due to the constant total enzyme concentration:

$$\frac{dln(\mu)}{dln(e_j)}=\frac{\partial ln(v_g)}{\partial n(e_j)}+\sum_{k\neq j}\frac{\partial ln(v_g)}{\partial n(e_k)}\cdot\frac{dln(e_k)}{dln(e_j)}=\frac{e_j}{e_{total}}+\sum_{k\neq j}\frac{e_k}{e_{total}}\cdot\frac{e_j}{e_k}\cdot\frac{de_k}{de_j}=\frac{e_j}{e_{total}}\cdot\left(1+\frac{\sum de_k}{de_j}\right)=0, \qquad \text{(Eq. 56)}$$

confirming that at constant total enzyme concentration, and also for this generalized unbranched pathway, the specific growth rate is now maximal with respect to variation in the concentration of any of the enzymes.

(Kacser and Beeby 1984; Heinrich et al. 1987) already considered growth rate per unit total enzyme, but also assumed that all other biomass components co-varied with the total concentration of enzyme (i.e. that $b_{00} = 0$). They further used the paradigm of a 'linear', *i.e.* sub-saturated unbranched metabolic pathway. We here generalized the analysis to any network (but see section 2.9.1) for which all e_j's can be optimized to become equal to:

$$e_j=_{all\ j}C_j^{\mu}\cdot e_{total}, \qquad \text{(Eq. 57)}$$

which, parenthetically, implies that the control of all enzymes on the

specific growth rate must be positive (see section 2.9.1).

2.8.2.2. The Edinburgh pathway at constant total enzyme and biomass concentrations. We shall illustrate these results for the simpler example of the Edinburgh pathway (Fig. 1A), for which we write for the change in flux upon up-modulation of the concentration of enzyme e_1:

$$\frac{dln(J)}{dln(e_1)} = C_1^J + C_2^J \cdot \frac{dln(e_2)}{dln(e_1)} \quad \text{(Eq. 58)}$$

When total enzyme concentration is constant:

$$de_1 = -de_2, \quad \text{(Eq. 59)}$$

and

$$\frac{dln(J)}{dln(e_1)} = C_1^J - C_2^J \cdot \frac{e_1}{e_2} \quad \text{(Eq. 60)}$$

At a smooth maximum $\frac{dln(J)}{dln(e_1)}$ should equal zero. This is consistent with the flux control summation law provided that the enzyme concentrations obey the following relationship:

$$\left(\frac{e_1}{C_1^J}\right)_{optimal} = \left(\frac{e_2}{C_2^J}\right)_{optimal} = e_{total}, \quad \text{(Eq. 61)}$$

in accordance with the more general equations derived above and by (Klipp and Heinrich 1999). One scenario for the optimization process is as follows: At fixed total enzyme concentration one may start the optimization at very low e_1, i.e., when enzyme 1 will have virtually all control, so that the ratio $\frac{e_1}{C_1^J}$ will be virtually equal to zero. Increasing the concentration of enzyme 1 and decreasing the concentration of e_2 by the same amount will decrease the flux control coefficient of the former and increase the ratio $\left(\frac{e_1}{C_1^J}\right)$ until it equals e_{total}. Meanwhile the other ratio $\frac{e_2}{C_2^J}$ was decreasing until it became equal to the total enzyme concentration, too. One here readily sees that the sum of the catalytic control coefficients can equal 1 whilst the enzyme concentrations are optimal with respect to maximal flux, provided that the total enzyme concentration is constant; there is now no inconsistency between the summation law of MCA and smooth optimality of the flux that is addressed. Somewhat surprisingly the possible difference between the enzymes in maximum rate per unit protein (which equals the catalytic rate constant) does not detract from this result: the enzyme catalyzing the more difficult reaction should just be expressed more to reach the optimum. Ribulose 1, 5-*bis*phosphate carboxylase (Rubisco) catalyzing CO_2 fixation may constitute an example (Raven 2013).

2.8.3. But: When inert biomass compensates to maintain biomass constant, there is no smooth optimum in the dependence of specific growth rate on enzyme concentrations

2.8.3.1. Arbitrary pathway at constant total enzyme and biomass concentrations. Living cells consist of more than enzymes. We shall denote the extra (non-enzyme) 'inert' biomass by b_0 and continue to assume that total biomass is constant and that compensation for any change in total enzyme level is through this inert biomass. The sum of dependencies of the specific growth rate on the enzyme activities then becomes:

$$\sum_j \frac{dln(\mu)}{dln(e_j)} = 1 - \sum_j \sum_{l \neq j} C_{kl}^{v_s} \cdot \frac{e_j}{e_l} \cdot \gamma_l - \varphi_e \cdot (1 - \gamma_0 - \gamma_e) = 1 - 0 - 0 = 1 \quad \text{(Eq. 62)}$$

This means that, if inert biomass compensates to keep total biomass constant, not all dependencies of the specific growth rate on enzyme concentrations can be at a smooth optimum.

2.8.3.2. The Edinburgh pathway at constant total biomass concentrations, inert biomass compensating. Defining $\beta > 0$ as the extent of the compensation of the change in enzyme level by the catalytically inert biomass (i.e. $\beta \stackrel{def}{=} -\frac{db_0}{de_1}$):

$$\frac{dln(J)}{dln(e_1)} = C_1^J + C_2^J \cdot \frac{e_1}{e_2} \cdot \frac{de_2}{de_1} = C_1^J - C_2^J \cdot \frac{e_1}{e_2} \cdot (1 - \beta) \quad \text{(Eq. 63)}$$

Because we here consider a decrease in inert biomass to compensate for an upward modulation of an enzyme, we write minus a positive factor; this in contrast to Eq. 52. Requiring optimality with respect to enzyme 1 and the validity of the summation law for the two catalytic flux control coefficients one finds for the control coefficient of enzyme 1:

$$C_1^J = \frac{e_1 \cdot (1 - \beta)}{e_{total} - \beta \cdot e_2} \quad \text{(Eq. 64)}$$

Also requiring optimality with respect to modulation of enzyme 2, one finds the analogous expression for the flux control coefficient of enzyme 2. Requiring the summation law to apply, one finds the following condition for the extent of compensation β:

$$\frac{e_1 \cdot (1 - \beta)}{e_t - \beta \cdot e_2} + \frac{e_2 \cdot (1 - \beta)}{e_t - \beta \cdot e_1} = 1 \quad \text{(Eq. 65)}$$

The left hand side of this equation is a monotonically decreasing function of β, which proves that $\beta = 0$ is the only solution. This implies that for smooth optimality to occur, all compensation must be exclusively between the enzymes, and that inert biomass should thereto be constant. This analysis shows that for as long as there is any compensation of the increase in enzyme concentration by reduction of the inert biomass concentration, the flux cannot become maximal with respect to the amount of either enzyme.

2.8.4. Sequential compensation by inert biomass and enzymes: down to a trade-off

When inert biomass is compensating fully, increases in the amount of either enzyme will be accompanied by a decrease in inert biomass concentration, down to a level of the latter that is minimally required for survival of the organisms (which, after all will need phospholipids for its membranes). When that lower limit of inert biomass has been achieved and b_o has become constant ($b_0 = b_{0.minimal}$), the flux can again become optimal with respect the variations in enzyme concentrations, provided that decreases in the levels of other enzymes compensate for the increase in level of the modulated enzyme. We conclude then that ultimately the enzyme concentrations can indeed become optimal with respect to flux, whilst the summation law of MCA is valid. In this situation the paradox is again resolved.

However, this conclusion assumes that the decrease in inert biomass concentration down to what is minimally required, comes without implication for fitness, until a 'hard' border ($b_0 = b_{0.minimal}$) is reached below which survival is impossible. In reality we expect there to be a trade-off, where a reduction in b_o, favored because then the enzyme concentrations and hence the specific growth rate can increase, is possible but is accompanied by a fitness cost that increases further with a further reduction in b_0. To illustrate the consequences, we define a fitness function ($F(J(e_1, e_2)),b)$) and require its dependence on enzyme e_1 (and e_2) to equal zero:

$$0 = \frac{\partial \ln(F)}{\partial \ln(J)} \cdot \frac{dln(J)}{dln(e_1)} + \frac{\partial \ln(F)}{\partial \ln(b)} \cdot \frac{dln(b)}{dln(e_1)}, \quad \text{(Eq. 66)}$$

whilst the fitness coefficients $\frac{\partial \ln(F)}{\partial \ln(J)}$ *and* $\frac{\partial \ln(F)}{\partial \ln(b)}$ are positive. We simplify the equation to:

$$\frac{dln(J)}{dln(e_1)} = \frac{\frac{\partial \ln(F)}{\partial \ln(b)}}{\frac{\partial \ln(F)}{\partial \ln(J)}} \cdot \frac{-dln(b)}{dln(e_1)} = \frac{\frac{\partial \ln(F)}{\partial \ln(b)}}{\frac{\partial \ln(F)}{\partial \ln(J)}} \cdot \frac{e_1}{b} \cdot \beta \quad \text{(Eq. 67)}$$

Since the fitness function depends positively on both flux and inert

biomass and $\beta > 0$, the dependence of the flux on the concentration of the enzymes must exceed zero. This reconfirms that whenever there is any compensation by a reduction in inert biomass, the enzyme concentrations cannot (both) be optimal with respect to any flux J, at least not in the sense of a smooth optimum.

2.9. Maintenance and branched pathways

2.9.1. No maximum in the dependence of specific growth rate on catabolic or anabolic enzymes if maintenance has a soft cost function

For branched pathways, the expression $(e_j)_{optimal} = C_j^{\mu} \cdot e_{total}$ is no longer valid (Klipp and Heinrich 1999). One can readily understand this by inspecting Fig. 1B: The maintenance process will exert a negative control on the specific growth rate. Because enzyme concentrations cannot become negative this means that the requirement for reaching the optimum (Eq. 54) cannot be met.

Optimization of the distribution of protein between the three enzyme groups in the Amsterdam pathway (Fig. 1B) at constant total enzyme concentration, should drive the maintenance enzyme levels to zero, thereby leading to the Edinburgh pathway (Fig. 1A) with the corresponding results. Here the optimum is not smooth in terms of the maintenance enzyme; it is a hard optimum at $e_m = 0$.

Importantly however, the Edinburg pathway does *not* represent the growth and persistence of organisms, as maintenance metabolism is essential (Hellingwerf et al., 1982; Kempes et al., 2017; van Bodegom 2007). The Amsterdam pathway (Fig. 1B) is more appropriate. Considering a variation of anabolic enzyme g, the optimality requires that (we use subscripts g, c and m, for growth = anabolism, catabolism and maintenance, respectively and assume that the maintenance enzyme level (e_m) remains constant):

$$\frac{dln(v_g)}{dln(e_g)} = C_g^{v_g} - C_c^{v_g} \cdot \frac{e_g}{e_c} \qquad \text{(Eq. 68)}$$

For maximal flux the result is thereby similar to that obtained for the Edinburgh pathway, except that the ratios are now not equal to the total enzyme concentration:

$$\frac{e_c}{C_c^{v_g}} = \frac{e_g}{C_g^{v_g}} = \frac{e_g + e_c}{1 + \left(-C_m^{v_g}\right)} < e_{total}, \qquad \text{(Eq. 69)}$$

with:

$$C_m^{v_g} < 0 \qquad \text{(Eq. 70)}$$

For all three enzymes this is indeed (Klipp and Heinrich 1999) different from the result obtained for a linear pathway. Yet, also in this case the growth rate is smooth maximal in terms of the concentrations of the catabolic and anabolic enzymes, and provided the sum of flux control coefficients is evaluated over the catabolic, the anabolic and the maintenance enzymes, that sum equals 1 and the paradox is resolved.

However, e_m may not be subject to a hard minimum, but rather to a softer one. Assuming a fitness function $F(v_g, v_m)$ to be maximal, where the fitness coefficients $\frac{\partial \ln(F)}{\partial \ln(v_m)}$ *and* $\frac{\partial \ln(F)}{\partial \ln(v_g)}$ are positive, we find:

$$\frac{dln(v_g)}{dln(e_j)} = \frac{\frac{\partial \ln(F)}{\partial \ln(v_m)}}{\frac{\partial \ln(F)}{\partial \ln(v_g)}} \cdot \frac{-dln(v_m)}{dln(e_j)}, \qquad \text{(Eq. 71)}$$

which again shows that in the optimum there need not be a smooth maximum of the specific growth rate as function of any of the enzymes whenever there is any compensation by lowered maintenance enzyme levels.

We conclude that optimization of specific growth rate by adjustment of the enzyme concentrations can only be complete if there is no compensation by adjustment of the biomass component of biomass, and then only in the case of a linear pathway from catabolic substrate to growth, i.e. with neglect of maintenance metabolism. This is not realistic.

2.9.2. Growth yield maximization

2.9.2.1. Summation law for yield constitutes no paradox. In balanced growth, the growth yield (y) is defined as the ratio of the growth rate (V_g) to the substrate consumption rate (V_c):

$$y \stackrel{\text{def}}{=} \frac{V_g}{V_c} = \frac{v_g}{v_c} \qquad \text{(Eq. 72)}$$

At balanced growth both v_g and v_c (the catabolic flux per cell) are time independent. The yield has no time dimension so that MCA's summation law for the yield reads:

$$\sum_{all\ js} C_{kj}^{y} = {}_{balanced\ growth}0 \qquad \text{(Eq. 73)}$$

This new law again shows that Metabolic Control Analysis can be extended to the control of any free variable in a dynamic system. This summation law also formally removes the paradox: in theory all the control coefficients on the yield could equal zero, and the yield in balanced growth could therefore be maximal with respect to all catalytic activities. Indeed, in the Edinburgh pathway (Fig. 1A), the yield is fixed, so that all yield control coefficients equal zero. Because they cannot vary, this does *not* correspond to the inverted parabola required for a proper smooth optimum (see the second of the Eqs. 18).

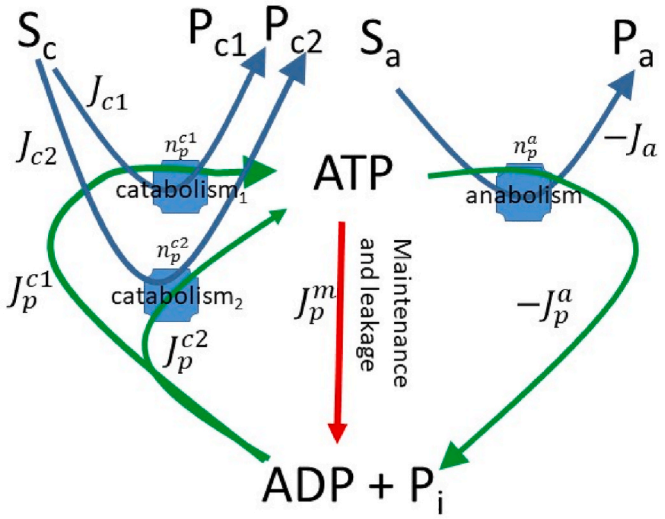

Fig. 2. Elaboration of Fig. 1B for Gibbs-energy (ATP) limited growth. The relevant intermediate between catabolism and anabolism is represented by the Gibbs energy of synthesis of ATP and the analysis is in terms of so-called Mosaic Non Equilibrium Thermodynamics (Westerhoff and Van Dam 1987). ATP is synthesized from ADP and phosphate (P_i) in a process that couples this conversion to the catabolism of the catabolic substrate S_c to the catabolic product P_{c1} or P_{c2}, at ATP/S_c stoichiometries of $n_p^{c_1}$ and $n_p^{c_2}$, respectively. Some of the ATP produced by catabolism is hydrolyzed at a stoichiometry n_p^a to ADP and phosphate whilst driving the anabolism of S_a into anabolic products P_a (e.g. biomass) in a process called 'growth' at rate $-J_a$. A third reaction (J_p^m) hydrolyzes ATP to ADP and phosphate and is called 'leakage' when it serves no function, or 'maintenance' when it serves the persistence of the living state (Westerhoff et al. 1982). The figure as shown can serve to illustrate the case of respirofermentative growth and the concept of 'overflow catabolism' (e.g. (Van Hoek, Van Dijken, and Pronk 1998)). By equating P_{c1} and P_{c2}, this figure can be made to represent 'gear shifting' when the two catabolic pathways have different ATP/S_c stoichiometries (Zhang and Westerhoff 2023). Since yield is taken with respect to catabolic substrate S_c, which is the same for both options, this does not affect the analysis in the text. Catabolism, growth and maintenance/leakage will be referred to by indices 'c', 'a', and 'm' in the text and equations.

2.9.2.2. Still not all individual yield control coefficients equal zero. As discussed above, the Edinburgh pathway is not a good model for microbial growth however, as it lacks maintenance metabolism. During balanced growth maintenance can consume more than half the ATP made in catabolism (and all that ATP in stationary phase) (Pirt 1982; Tempest and Neijssel 1984). The Amsterdam pathway (Fig. 1B) may therefore be a better model for microbial growth.

For illustration purposes we shall resort to a simplified description in terms of a combination of Mosaic Non Equilibrium Thermodynamics (Westerhoff et al., 1982) and Metabolic Control Analysis (Westerhoff and Van Dam 1987). As also illustrated by Fig. 2, this takes the normalized Gibbs energy of ATP synthesis ($\frac{\Delta G_p}{R \cdot T}$) as the intermediate, which is called 'x' and dimensionless. Fig. 2 is thereby an elaboration of the Amsterdam pathway (Fig. 1B) for when growth is 'energy limited', which means that ATP and not a carbon or a nitrogen intermediate is the relevant intermediate between catabolism and anabolism (Westerhoff and Van Dam 1987). We first consider the version of the model where only one of the two catabolic processes shown is active. At the steady state, which corresponds to the condition of balanced growth (see above) hence to no change in intracellular ATP concentrations with time, the specific growth rate $-J_a = \mu$ (in this section we use $-J_a$ rather than μ, and more generally J's for the fluxes, because we here venture into the area of non equilibrium thermodynamics and do not wish to lose correspondence with that area and with the equations derived in (Westerhoff and Van Dam 1987; Westerhoff et al., 1982; Westerhoff et al. 1983)) is related to the other fluxes and the stoichiometries by:

$$y = \frac{-J_a}{J_c} = \frac{n_p^c}{n_p^a} - \frac{J_p^m}{n_p^a \cdot J_c} = \frac{n_p^c}{n_p^a}\left(1 - \frac{J_p^m(x)}{n_p^c \cdot J_c(x)}\right) \quad \text{(Eq. 74)}$$

The actual growth yield varies with how anabolism and maintenance depend on the the metabolic intermediate x, generally in a complex way. The complexity can be reduced by introducing the elasticity coefficients (Burns et al., 1985) ε_x^j of the three processes with respect to that Gibbs energy difference. It is defined as the derivative of the logarithm of the corresponding process rate v_j with respect to that Gibbs energy of ATP synthesis (Westerhoff and Van Dam 1987):

$$\varepsilon_x^j \stackrel{\text{def}}{=} \frac{\partial \ln |v_j|}{\partial x} \quad \text{(Eq. 75)}$$

Using the flux-control connectivity and summation laws, the three flux control coefficients with respect to the anabolic flux can now be expressed into the elasticity coefficients ((Westerhoff and Van Dam 1987), page 446), and the same for the control coefficients with respect to the catabolic flux. Taking the difference between the anabolic and the catabolic flux control coefficients as the control coefficient on the yield, and setting the stoichiometries to 1 for simplicity, one obtains

$$C_c^y = \frac{(1-y)\cdot(\varepsilon_X^a - \varepsilon_X^m)}{-\varepsilon_X^c + y\cdot\varepsilon_X^a + (1-y)\cdot\varepsilon_X^m} =_{y=1} 0 \quad \text{(Eq. 76)}$$

$$C_a^y = \frac{(1-y)\cdot(\varepsilon_X^m - \varepsilon_X^c)}{-\varepsilon_X^c + y\cdot\varepsilon_X^a + (1-y)\cdot\varepsilon_X^m} =_{y=1} 0 \quad \text{(Eq. 77)}$$

$$C_m^y = -\frac{(1-y)\cdot(\varepsilon_X^a - \varepsilon_X^c)}{-\varepsilon_X^c + y\cdot\varepsilon_X^a + (1-y)\cdot\varepsilon_X^m} =_{y=1} 0 \quad \text{(Eq. 78)}$$

confirming that the sum of the yield-control coefficients always equals 0. Even more so, in the absence of leak and maintenance processes, $y = 1$ and all control coefficients on the yield are zero, consistent with optimality throughout.

However, although the paradox between summation law and optimality herewith disappears, there still appears to be a paradox between the flux control coefficients expected by MCA and optimality: whenever there is maintenance and the yield does not equal 1, the individual yield control coefficients are not equal to zero. Should optimization then be able to modulate the catabolism, anabolism and maintenance independently, no smooth maximum could be obtained.

Maintenance metabolism is important for cell survival (see section 9.2.2.2). If we consider the situation where the amount of enzyme engaged in maintenance metabolism has reached its minimum and is thereby constant. Then the absence of a variation of the yield with the amount of anabolic and catabolic enzyme in a way consistent with the summation law for yield control coefficients and at constant total constant enzyme concentration and negligible variation in maintenance enzymes, requires (Eq. 69):

$$\frac{C_a^y}{e_a} = \frac{C_c^y}{e_c} \quad \text{(Eq. 79)}$$

As these conditions can be met by variations in e_a and e_c, it is possible that yield is optimal with respect to the variation of the catabolic and anabolic enzyme levels at constant total enzyme concentration and constant maintenance enzyme levels.

If however the compensation is in terms of the maintenance metabolism only, then the condition is:

$$\frac{C_a^y}{e_a} = \frac{C_m^y}{e_m} \quad \text{(Eq. 80)}$$

which is impossible as the maintenance exerts a negative control on the yield.

We conclude that the growth yield can be optimal with respect to the variation in enzyme levels at total biomass concentration if it is the other enzymes and not the maintenance enzyme that compensates. The evolutionary scenario here could be a decrease in maintenance enzyme until it has reached its minimum followed by an optimization of the anabolic and catabolic enzyme levels. However we conjecture (in line with the above examples of combined optimizations) that in the more realistic case where fitness is a function of both yield and maintenance metabolism, the optimal state is not at the enzyme concentrations that are optimal for yield alone.

2.10. FBA

2.10.1. FBA and the paradox. A standard FBA procedure has a fixed catabolic flux (see above) due to an upper bound, set for that flux. It then finds the flux pattern that maximizes some output flux, often the specific growth rate (Schuster et al. 2008). Since the yield equals the specific growth rate divided by the specific catabolic flux (Eq (74)), the fixation of the catabolic flux implies that FBA simultaneously optimizes specific growth rate and growth yield. As the setting of the bound for the catabolic flux is often arbitrary, FBA effectively optimizes growth yield (Schuster et al. 2008) (for an actual example see (Fell and Small 1986)). Because MCA's summation law for yield arrives at a zero rather than a 1 (Eq. (73)), there is no direct paradox between optimization and FBA. However, there is an issue concerning the type of optimum describe by FBA: it is usually not the 'soft optimum' that we considered in the above discussions.

2.10.2. FBA and hard optima. The fixed catabolic flux of FBA (see above) corresponds to the reaction rate of the catabolic reaction being unresponsive to changes in the concentration of the intermediary metabolite X of Fig. 1, i.e. to its elasticity for X equaling zero. Then:

$$C_c^{J_c} = \frac{y\cdot\varepsilon_X^a + (1-y)\cdot\varepsilon_X^m}{y\cdot\varepsilon_X^a + (1-y)\cdot\varepsilon_X^m} = 1, \quad \text{(Eq. 81)}$$

confirming that in this FBA setting catabolism is in full control of the catabolic flux whilst neither anabolism nor maintenance exert control over it. Also growth is then largely controlled by catabolism and even completely so when there is no maintenance ($y = 1$):

$$C_c^{-J_a} = \frac{\varepsilon_X^a}{y\cdot\varepsilon_X^a + (1-y)\cdot\varepsilon_X^m} =_{y=1} 1 \quad \text{(Eq. 82)}$$

$$C_a^{-J_a} = \frac{(1-y)\cdot\varepsilon_X^m}{y\cdot\varepsilon_X^a + (1-y)\cdot\varepsilon_X^m} =_{y=1} 0 \quad \text{(Eq. 83)}$$

$$C_m^{-J_a} = \frac{-(1-j)\cdot\varepsilon_X^a}{y\cdot\varepsilon_X^a + (1-y)\cdot\varepsilon_X^m} =_{y=1} 0 \quad \text{(Eq. 84)}$$

The summation laws of MCA are still met: The sum of these flux control coefficients with respect to the specific growth rate $-J_a$ equals 1.

Should one now vary the upper bound set to the catabolic flux, the growth rate would increase (virtually) in proportion. The maximum flux is reached because there is a restriction to the increase in the catabolic enzymes: This is what we defined in section 2.4 as a hard optimum. It is consistent with the summation law of MCA. Also the yield is controlled by the catabolic flux bound: also in terms of yield this is a hard optimum, determined by the maximum of e_c.

2.10.3. FBA and a smooth optimum through gear shifting. Allowing two parallel catabolic pathways, such as depicted in Fig. 2, the specific growth rate $-J_a$ is related to the other fluxes and the stoichiometries by:

$$-J_a = \frac{J_{c1}\cdot n_p^{c1} + J_{c2}\cdot n_p^{c2} - J_p^m}{n_p^a} \quad \text{(Eq. 85)}$$

Fixing the catabolic flux at (e.g.) 1, FBA then choses a flux pattern that delivers the highest $-J_a$. If $n_p^{c1} > n_p^{c2}$ this will be pathway 2. In principle, the FBA should also reduce the uncoupled ATP hydrolysis to zero (but see below), so that the yield (y) becomes equal to the ratio of the two stoichiometries:

$$y_{FBA} \stackrel{\text{def}}{=} \frac{-J_{aFBA}}{J_{c.max}} = \frac{n_p^{c2}}{n_p^a} - \frac{J_p^m}{n_p^a\cdot J_c} \quad \text{(Eq. 86)}$$

According to the biochemistry known for anabolism, microbial growth from inorganic substrates requires approximately 27 mmol ATP per gram dryweight. Consequently, $n_p^a \cong 0.027$ mol ATP/gram dryweight (Stouthamer 1973). 42% of dryweight of *E. coli* is Carbon (Fagerbakke et al. 1996). Consequently, this n_p^a should also correspond to 0.77 mol ATP/C-mol biomass (this number is approximate only: using the numbers of (Westerhoff and Van Dam 1987) that 1 g of biomass contains 41 mmol Carbon = 0.49 g, this becomes 0.66 mol ATP/C-mol biomass).

Predecessors to Flux Balance Analysts computed the number of ATP molecules produced per unit catabolism (n_p^{c2}) (e.g. $n_p^{c2} = 0.33$ *mole ofATP per Cmole of glucose converted to lactate*), and then found that experimental growth yields were approximately half of $\frac{n_p^{c2}}{n_p^a}$ (Hellingwerf et al., 1982). In order to fit these results, most FBA does not allow its optimization to remove or reduce maintenance metabolism but rather considers it to be a fixed property, which has been fitted to the experimental data (Edwards et al. 2001; Ulas et al., 2012; Liu and Westerhoff 2023).

The reaction stoichiometries n_p^{c2} and n_p^a are fixed numbers in FBA. Asking for a smooth optimum (see above) in the concentration of an enzyme would therefore require a parabolic dependence of the growth rate on the activity (or substrate) of the substrate transporter or catabolic enzyme with most flux control. This is neither likely nor the perspective of FBA however, as FBA is devoid of kinetics. The optimality of the growth rate must therefore derive from the substrate consumption flux hitting a hard border (its preset bound) in the FBA: this is a 'hard optimum' in the sense defined above. Notwithstanding the '*maximal*' growth rate suggested by the FBA, the growth rate *can* then be increased, i.e. by increasing the upper bound set for catabolism: Accordingly, it is not the growth rate but the growth yield that is then maximal in FBA.

By repeating this procedure at various magnitudes of the catabolic flux, FBA may deliver a smooth optimum however. If for instance the activity of pathway 2 of the catabolism in Fig. 2 is bounded by a fixed V_{max} (bound) $= J_{c_2\ max}$ one would find a maximum growth rate of $-J_{aFBA} = \frac{n_p^{c2}}{n_p^a}\cdot J_c - \frac{J_p^m}{n_p^a}$ at catabolic fluxes lower than this $J_{c_2\ max}$ and $-J_{aFBA} = \frac{n_p^{c2}}{n_p^a}\cdot J_{c_2\ max} + \frac{n_p^{c1}}{n_p^a}\cdot(J_c - J_{c_2\ max}) - \frac{J_p^m}{n_p^a}$ for $J_c > J_{c_2\ max}$. Consequently also the growth yield changes: $y_{FBA} \stackrel{\text{def}}{=} \frac{-J_{aFBA}}{J_c} \leq 0$ (i.e. negative or, realistically, no growth rate) for $J_c < J_p^m\cdot\frac{n_p^a}{n_p^{c1}}$. It then increases to $0 < \frac{n_p^{c2}}{n_p^a} - \frac{J_p^m}{n_p^a\cdot J_c} \leq 0$ for $J_p^m\cdot\frac{n_p^a}{n_p^{c1}} < J_c < J_{c_2\ max}$ and subsequently decreases again, ultimately to $\frac{n_p^{c2} - n_p^{c1}}{n_p^a}\cdot\frac{J_{c_2\ max}}{J_c} + \frac{n_p^{c1}}{n_p^a} - \frac{J_p^m}{n_p^a\cdot J_c}$. Then the growth yield should go through a smooth optimum, with an optimal value for the upper bound of catabolism. The respirofermentative growth of *S. cerevisiae* (Van Hoek, Van Dijken, and Pronk 1998) may be an example of this case. If both catabolic routes have the same beginning and end, the phenomenon is called gear shifting; it has indeed been shown that the yield goes through a maximum as the stoichiometry is increased (Zhang and Westerhoff, 2023).

2.11. The paradox revisited

The paradox we wish to address stemmed from the summation law of MCA specifying that the sum of the flux control coefficients over all enzymes of a network must equal 1. In its original form the flux control coefficient for which this summation law holds was defined as (the 'sensitivity coefficient') (Kacser and Burns 1973; Westerhoff et al. 1984; Groen et al., 1982):

$$C_j^J \stackrel{\text{def}}{=} \lim_{\delta e_j \to 0} \frac{\delta J}{J} \Big/ \frac{\delta e_j}{e_j} = \frac{dln|J|}{dln(e_j)} \quad \text{(Eq. 87)}$$

To Kacser and Burns and to us this then seemed natural, as we were thinking about biochemical pathways as if *in vitro*, such that we could titrate one enzyme. In such a (thought) experiment, the other enzymes would naturally remain at the same concentration. Indeed, this is how Kacser analyzed the experiments by (Flint et al., 1981) in which enzyme dosage was varied by using heterokaryons of *N. crassa*: he did not take on board that in such experiments the other enzymes in the network with flux control might vary as well. Later, when developing and applying (Snoep et al., 2002) hierarchical control analysis, which does accommodate changes in gene expression, we demonstrated the quantitative importance of this distinction.

Heinrich & Rapoport who also focused on linear pathways *in vitro* or in erythrocytes where gene expression is not adaptive, defined the same coefficient as ('control strength'):

$$C_j^J \stackrel{\text{def}}{=} \frac{\partial v_g}{\partial v_i} \quad \text{(Eq. 88)}$$

Here v_g referred to the steady state flux and v_i to the instantaneous reaction rate through the same step j in the linear enzymatic chain. As the authors considered unbranched pathways, this definition is equivalent to:

$$C_j^J \stackrel{\text{def}}{=} \frac{\partial ln v_g}{\partial ln v_i} \quad \text{(Eq. 89)}$$

Because v_i and v_g are equal (at least at steady state), this definition was confusing and mathematically contestable (Schuster and Heinrich 1992). This issue was resolved by the definition (Heinrich and Rapoport 1974) that became the consensus (Burns et al., 1985):

$$C_j^J \stackrel{\text{def}}{=} \left(\frac{\partial ln|v_g|}{\partial p_i}\right)_{steady\ state} \Big/ \left(\frac{\partial ln|v_j|}{\partial p_j}\right)_{fixed\ substrate\ and\ product\ concentrations}, \quad \text{(Eq. 90)}$$

where p_j refers to a parameter that specifically affects reaction j.

However also this definition was ambiguous: it was insufficiently clear what should be held constant. In particular it was unclear whether the parameter p_j could actually be the concentration of enzyme *j* just as well as the catalytic rate constant k_j and whether the concentrations of all the other enzymes (or if k_j was modulated, of all enzymes) should be held constant.

The importance of the difference, which was not clear to (Kacser and Burns 1973) (we quote 'The change in the rate, caused by the change in inhibitor, can always be thought of as equivalent to some change in the concentration of the enzyme') was perhaps first discussed by (Schuster and Heinrich 1992). It independently emerged in the study of the control by the phosphotransferase system in *E. coli* with reaction steps consisting of the transfer of a phosphoryl group between two enzymes. When flux control was defined in terms of enzyme concentrations, the sum of the flux control coefficients could become as high as 2, whilst when defined in terms of catalytic constants the sum remains 1 (Vandam et al., 1993). Subsequently it became consensus that flux control coefficients should be defined in terms of modulation of the catalytic rate constants (Kholodenko et al., 1995). This definition also settled the issue of the variation of the enzyme concentrations: these should be kept constant. Only in 'ideal' metabolic pathways without alterations in gene expression, modulation of enzyme concentration can be a substitute for modulation of the catalytic rate constant (Schuster and Heinrich 1992; Kholodenko et al., 1995). The dependence on the enzyme concentrations should be better classified as 'sensitivity coefficients' or (because this is too generic) 'enzyme-control coefficients'. Should we wish to emphasize the difference, the control coefficients may be called the 'catalytic control coefficients'.

This distinction between the flux control coefficient and the enzyme-control coefficients is important for the paradox we here address. Following the early interpretations of the flux control coefficients cited above, the enzyme control coefficient was taken to be the same as the catalytic control coefficient. The flux control summation theorem was thereby taken to mean that the sum of the enzyme-flux control coefficients should equal 1:

$$\sum_j \frac{dln|J|}{dln(e_j)} = 1, \qquad \text{(Eq. 91)}$$

On the other hand optimality of microbial growth was taken to mean that there should be a maximum in the dependence of the growth rate on the concentration of any of its enzymes, with as consequence:

$$\sum_j \frac{dln|J|}{d\ ln(e_j)} = \sum_j 0 = 0 \qquad \text{(Eq. 92)}$$

Now that we have reviewed the differences between the two types of control coefficient, it is clear that the resolution of the paradox merely resides in the difference between the two types. But what is this difference then, and do the differences always add up to 1 in a summation over all enzymes? Or are there other options for resolving the paradox? The possibilities were that:

(i) optimality should indeed be interpreted as a smooth maximum, but:
  a. the sum of the control coefficients for growth rate does equal zero
  b. some other property than growth rate should be maximal and:
    i. its sum differs from 0, but:
      1. its logarithmic derivative with respect to any $\ln(k_{cat,j})$ differs sufficiently from the logarithmic derivative with respect to $\ln(e_j)$ for the summed difference to equal 1
      2. the derivatives with respect to $\ln(e_j)$ are interdependent so that their sum equals zero
    ii. its summation does equal 0

(ii) the optimum is due to the enzyme levels being maximal: a 'hard' maximum

With respect to (i) we showed that, should growth rate be interpreted as population growth rate the sum would much exceed 1, eliminating option a. We then examined which properties might be alternative candidates for optimization and found the specific growth rate, cell growth rate, growth yield, and thermodynamic efficiency. We developed a more general proof for the summation law and showed that the sum of the control coefficients equal 1, 1, 0, and 0 for these four properties, respectively. We further confined the analysis to specific growth rate and yield.

With respect to the specific growth rate we reviewed that if the enzyme concentrations can be varied independently of one another, the sum dependence on the enzyme levels should equal 1, leaving the second sub-option ((i).b.i.2) as the remaining possibility. We then reviewed that if the variation in enzyme levels were restricted by the total enzyme concentration remaining constant, the dependence of specific growth rate on enzyme levels could equal zero, and be consistent with the summation law for specific growth rates. If total biomass needs to be constant and compensation also proceeds through reduction in the inert biomass component (such as lipids or carbohydrates) however, then the dependence on enzyme levels should not quite equal zero. In realistic cases of microbial growth there is substantial maintenance metabolism. We showed that his makes it impossible for the specific growth rate to be quite optimal with respect to variation of all the enzyme concentrations. Maintenance should also be subject to optimization, with a different relationship to fitness.

With respect to yield, we found that, as living organisms have maintenance metabolism, the growth yield can be subject to optimization. A smooth optimum may then occur in the yield when they have alternative catabolic pathways with different stoichiometries and maximum fluxes. Also here however, optimization of maintenance is likely to frustrate this option.

We finally examined option (ii) and showed that when the activity of an enzyme limiting the specific growth rate cannot increase any further for any reason, the optimum may indeed be a 'hard optimum', and thereby evade the paradox with MCA's summation law. This type of optimum is frequent in FBA.

3. Discussion

In this paper we have shown that the concept of optimality of microbial growth with respect to the enzyme expression levels is not in conflict with MCA's law of a total flux control of 1. The main reasons are that (i) optimality of the specific growth rate is addressed by the enzyme control coefficients ('sensitivity coefficients') (Schuster and Heinrich 1992) whereas the summation law is in terms of the 'catalytic' control coefficients, and (ii) if there are constraints that make the enzyme levels interdependent, the magnitudes of these two types of coefficients can differ.

The difference between the two types of control coefficients is minor when analyzing exclusively metabolic pathways as if *in vitro*, but does count when addressing variability of enzyme concentrations such as in variable gene expression (Westerhoff et al., 1990; He et al. 2013; Snoep et al., 2002) and in evolution. If one requires that total enzyme concentration be constant, there must be regulated gene expression achieving the compensation. Then the difference between the two types of control coefficients may matter therefore, with the result that there are two different summation laws for flux control. The one sums the catalytic flux control coefficients over all enzymes and finds the result of 1, whilst the other sums the enzyme (-concentration based) flux control coefficients and finds something less than 1 and potentially zero.

(Brown 1991) proposed that a constant total protein concentration might be an evolutionary constraint and that thereby the sum of the enzyme control coefficients might equal 0, which may well correspond to one of the general cases derived by (Schuster 1996). In their discussion of evolutionary optimization (Kacser and Beeby 1984) had already shown that if they assumed total enzyme concentration to be constant,

the sum of the enzyme control coefficients with respect to specific growth rate should equal 0 rather than 1 in the state of maximal specific growth rate. (Heinrich et al. 1987) showed that at constant total enzyme concentration, the optimization led to a reduction of the flux control coefficient by the fraction of total enzyme concentration made up by the modulated enzyme (they assumed that the cell volume is strictly proportional to the cell's protein content). When summing their expressions over all enzymes one obtains the usual sum of 1 minus 1, i.e. zero.

The analyses by (Kacser and Beeby 1984; Heinrich et al. 1987) considered growth rate per unit total enzyme, without much discussing the possible covariance of biomass components other than the enzymes (such as I did above), and used the paradigm of a linear sub-saturated pathway. (Klipp and Heinrich 1999) generalized the analysis to more complex pathways.

In this paper we did not only review (and essentially reproduce) the work by these authors, but also came upon two new and probably important ramifications. We had come across the first when we could not find convincing evidence that total enzyme concentration *is* always (precisely) constant during evolutionary optimization. In at least one case where we made ourselves a witness of evolution *in vitro*, biomass did change during evolution, not only qualitatively but also quantitatively, leading to a 'super micro-organism' (Groeneveld et al. 2009)). In the present paper we then found for unbranched pathways where there was either (i) no compensation so that biomass just increased as an enzyme was overexpressed, or (ii) there was constant biomass but the compensation of the increase in enzyme partly or wholly consisted of a decrease in inert biomass (such as carbohydrates in the cell wall, membrane lipids, or glycogen storage), the enzyme-control coefficients should *not* add up to zero. We showed that they might actually sum up to 0.45, meaning that at least some enzymes should not be present at their calculated smooth optimum concentration.

The second ramification emerged when we realized that the exception status of branched pathways (Klipp and Heinrich 1999) should be important for microbial growth optimization. This is because maintenance metabolism, which is both substantial and essential for microbial fitness (Pirt 1982; Tempest and Neijssel 1984; van Bodegom 2007; Kempes et al., 2017), corresponds to a branch away from the linear pathway from catabolism to anabolism and growth (Figs. 1B and 2). Some cellular proteins catalyze reactions that are important for the maintenance of the living state. These include 'maintenance' enzymes that maintain ion gradients, keep the proteins in their most active confirmation, or assist in chemotaxis. Due to their consumption of ATP, these enzymes mostly exercise a negative control on the specific growth rate. From the specific growth rate perspective, their optimum concentration for maximum specific growth rate should thereby be zero. Yet, because total fitness also depends positively and more directly on them than just through that specific growth rate, maintenance must be substantial. Actual optimization for maximum specific growth rate will be a trade-off between increases in anabolic and catabolic proteins in order to increase growth rate on the one hand, and decreases in anabolic and catabolic protein in order to enable the synthesis of proteins involved in maintenance, on the other hand. Consequently, none of the enzyme control coefficients with respect to the specific growth rate will equal zero in the state that is optimal in terms of maximum specific growth rate alone.

And then there is the issue that, depending on the conditions, evolution may maximize growth yield rather than specific growth rate. As the sum of the MCA's control coefficients on yield equals 0, the paradox that we recognized for specific growth rate does not exist for yield. Due to the existence of maintenance however, the enzyme control coefficients on yield will be positive for anabolic enzymes and negative for maintenance enzymes. Again because maintenance and yield will both be important for fitness: one should not expect the enzyme concentrations to be optimal for the growth yield, at least not in the sense of a smooth optimum.

This brings us to a third major issue: it may not always be possible to increase the concentration of catabolic or anabolic enzymes; they may be subject to a hard maximum. Then the specific growth rate or yield cannot increase either. This is what we have called a 'hard optimum'. Because FBA often uses upper bounds for its process rates, optimal states predicted by FBA are often such hard optima. However, if there are catabolic routes with different stoichiometries of, e.g. ATP synthesis, organisms may shift to lower gear (Zhang and Westerhoff 2023; Mondeel et al., 2016) at high substrate concentrations, so that the yield as function of the catabolic substrate moves through an optimum. Then FBA may deliver smooth optima.

Flux control coefficients can be small. Indeed, in hierarchical control analyses (Westerhoff et al., 1990; Kahn and Westerhoff 1991), it has been shown that control at the metabolic level can be ameliorated substantially by control through gene expression or signal transduction (Jensen et al., 1999; Snoep et al., 2002; Kahn and Westerhoff 1991). Moreover, an increase in the expression level of an enzyme can have a smaller effect than the activation of its activity because of concomitant reduction of the ribosome concentration; ribosome activity is a major determinant of the specific growth rate (Molenaar et al., 2009). Competition of mRNAs for those ribosomes (Jensen et al. 1993a, 1993b; Jensen et al. 1993a, 1993b), or competition of proteins for cell (Brown 1991) or membrane (Jensen et al. 1993a, 1993b; Groeneveld et al. 2009) space, may also limit the effect of over-expression of genes on the specific growth rate (Snoep et al., 1995).

The time dependent summation laws we here introduced are new for the specific growth rate, growth yield and thermodynamic efficiency. The laws for flux and concentrations have been proven before, but with different methods. One of the previous methods used the paradigm that system behavior should remain the same except for an acceleration by a factor if all enzyme activities are increased by that same factor (Acerenza et al. 1989). Another methodology recognized that state variables are homogeneous functions of the enzyme activities and inverse time (Westerhoff and Van Dam 1987; Giersch 1988). And yet another methodology employed implicit differentiation (Heinrich and Rapoport 1974; Reder 1988; Kahn and Westerhoff 1991). All these methods are related and use the concept that functions of the dynamic systems studied, depend on all rate constants and do not depend on parameters with time dimensionalities that differ from -1 (Westerhoff 2023). Both the first of these three methodologies and the new methodology used here, have led to summation laws for time dependent control coefficients, but only the method used here has been applied (here) to efficiency and yield.

A remaining riddle in FBA is the conclusion (Edwards and Palsson 2000) that the specific growth rates of various metabolic variants of *E. coli* observed experimentally, validated FBA predictions for maximal growth yield. This conclusion was in apparent conflict with the conclusion that had been reached when comparing between actual and theoretical growth yields, which was that experimental growth yields were not maximal at all. The theoretical growth yield had been calculated by dividing the $Y_{ATP.max.theoretical}$ *i.e.* the amount of ATP that should be required to synthesize a new cell (Stouthamer 1973) by the amount of ATP the organism should produce in catabolism. Actual experimental growth yields are only approximately half this theoretical maximum (Roels 1980; Hellingwerf et al., 1982; Westerhoff and Van Dam 1987; Verduyn et al., 1991; Heijnen 1991; Stouthamer 1976). The difference between experimental and theoretical growth yield may again reflect that neither growth rate nor growth yield are of sole importance to microorganisms (Westerhoff and Van Dam 1987): Microorganisms expend some of their metabolic free energy in 'maintenance' processes, which enhance viability at the cost of growth (Hellingwerf et al., 1982).

This FBA riddle is partly resolved by realizing that in the FBA calculations of 'optimal' flux patterns, a growth-rate independent 'maintenance' process at constant flux is commonly added (Edwards and Palsson 2000; Herrgard et al., 2008). This maintenance pathway is then not considered for optimization (hence minimization), for reasons that are barely made explicit. Should the maintenance process be considered

for optimization with maximum growth yield as sole criterion, it should disappear and the growth yield predicted by FBA should become some 40% larger than what is observed experimentally and should then correspond much more closely to what is expected from the $Y_{ATP.\ max.\ theoretical}$. It is more likely that there is a trade-off between maintenance and anabolism: A reasonable interpretation is that to be able to grow, cells must also be able to survive their cell replication cycle during which they are subject to multiple damage reactions. Because damage is necessarily thermodynamically downhill, its repair comes at the cost of ATP hydrolysis (such as the maintenance reaction in Fig. 2). Rapidly growing cells may be subject to more damage, so that part of the maintenance may be growth rate dependent (Hellingwerf et al., 1982). Maintenance processes may include compensation for passive leakage of ions across the bacterial plasma membrane (Maeda et al., 2019), repair of damaged macromolecules (Stan et al. 2022), and cell motility (van Bodegom 2007). A case in point is the proton mediated free-energy transduction involving electric potentials around 150 mV (Westerhoff and Van Dam 1987) across membranes some 4 nm in dielectric thickness (Andersen and Koeppe, 2007). This corresponds to an electric field of approximately 40 MV/m, exceeding the field strength of dielectric breakdown in air and oils (4 MV/m; admittedly this is the breakdown for electrons jumping between electrodes; the reversible electrical breakdown voltage of lipid bilayer membranes in 1 M KCl was found to be closer to 1 V (Benz et al. 1979)). These free-energy transducing membranes may thereby be subject to continuous 'nanoscopic thunderstorms', leading to a substantial leakage of protons and other ions across them, as substantiated in assessments of the non-ohmic conductance across mitochondrial inner membranes (Nobes et al., 1990). During evolution of many species, the advantage of the high ATP yields of oxidative phosphorylation (as compared to those of fermentation) appears to have outweighed this loss that may be inherent to the chemiosmotic coupling mechanism. Yet, the phenomenon should still lead to a lower growth yield than expected theoretically for when the proton leak is absent.

These considerations may provide an explicit basis for the strategy that is mainstream in FBA i.e. that of taking maintenance metabolism into account. It is less obvious however, that maintenance metabolism should proceed at a constant flux; there are many reports of regulation of maintenance processes (Kempes et al., 2017). The conclusion drawn then should be that microbial growth appears to be optimal for some combination of growth yield and maintenance of the living state, as we have discussed in some more detail above. The assumed invariability of maintenance warrants a critical assessment. And even then, the discussion is still open whether the optimization does not have an even more complex objective than growth yield and maintenance. Comparisons of actual growth rates, yields, and thermodynamic efficiencies have suggested that microorganisms do not just aim at maximum growth rate or growth yield but seek a compromise between maximum growth rate and maximal thermodynamic efficiency or yield in ways that sacrifice Gibbs energy, thermodynamic efficiency, and even material yield. (Westerhoff et al. 1983; Abudukelimu et al., 2017; Mondeel et al., 2016; MacLean 2008) discussed that depending on cell density there may indeed be a rate-yield trade-off. The issue has a longer history in the identification of so-called r-selection and K-selection by (MacArthur and Wilson 2001) in their theory of island biogeography. In a microbial view, yield maximization should be obtained in a repeated culturing of single cells on plates or in oil water emulsions (Bachmann et al., 2016), leading to colonies that stop growth when their local substrate is depleted (Westerhoff et al. 1983), whilst rate maximization may occur in well-stirred suspensions. (Schuster et al. 2008) reviewed evidence for maximization of yield, but often more in terms of metabolic yield (such as ethanol per glucose in *S. cerevisiae*) than growth yield, adding further complication. This suggests that either rate (r) and yield (K) optimization may occur depending on the dynamics of the selection conditions, and on the various dynamic limitations in nutrients (van Pelt-KleinJan et al. 2021). An optimal state may also need to be ready for anticipated changes in the conditions around the cell. The involvement of much of the genome in signal transduction may testify to this (Bruggeman et al., 2000; Westerhoff et al., 2014). Depending on what the actual growth conditions select for and constrain, one may expect even more complex optimization mechanisms (Simeonidis et al., 2010; Richard et al., 1996; Bachmann et al., 2016; Rabbers et al., 2021; Grigaitis and Teusink 2022; Maeda et al., 2019). A further integration between MCA and FBA methodologies, with further analysis of the complexity of evolutionary fitness, may help address these issues.

We have here discussed the possibility of microbial growth being optimal for growth rate or growth yield, in view of the underlying principles of MCA. These principles constitute the network basis for efficient or mechanistic causality. This leaves the question whether microbial growth is actually optimal. In a parallel paper Bruggeman and colleagues show how the final cause of optimality, the *telos* or objective, could be served by the organisms by using elementary flux modes (Bruggeman et al., 2023). They report that indeed this teleological cause of optimality seems to have shaped the metabolism (as well as the regulation thereof) of microorganisms such as *E. coli* and *S. cerevisiae*.

Declaration of competing interest

The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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