

Control of specific growth rate in *Saccharomyces cerevisiae*

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In this contribution we resolve the long-standing dispute whether or not the Monod constant (K_S), describing the overall affinity of an organism for its growth-limiting substrate, can be related to the affinity of the transporter for that substrate (K_M). We show how this can be done via the control of the transporter on the specific growth rate; they are identical if the transport step has full control. The analysis leads to the counter-intuitive result that the affinity of an organism for its substrate is expected to be higher than the affinity of the enzyme that facilitates its transport. Experimentally, we show this indeed to be the case for the yeast *Saccharomyces cerevisiae*, for which we determined a K_M value for glucose more than two times higher than the K_S value in glucose-limited chemostat cultures. Moreover, we calculated that at glucose concentrations of 0.03 and 0.29 mM, the transport step controls the specific growth rate at 78 and 49%, respectively.

INTRODUCTION

In the classic publication (Monod, 1949) in which Monod proposed his famous equation for specific growth rate (μ) dependency on substrate concentration (equation 1), he suggested, realizing the similarity with the Michaelis–Menten equation, that ‘the K_S value should be expected to bear some more or less distant relation to the apparent dissociation constant of the enzyme involved in the first step of the breakdown of a given compound’.

$$\mu = \frac{\mu_{\max} \cdot S}{S + K_S} \quad (1)$$

Monod appeared to be very careful in this statement, and rightly so for the mechanistic interpretation of an empirical constant that is dependent on the systemic behaviour of thousands of reactions is not simple and lies at the core of the relatively new research field of systems biology.

Whereas the Michaelis–Menten equation was derived on the basis of an enzyme kinetic mechanism and its parameters have a mechanistic interpretation, the Monod equation is purely empirical. Thus the Monod constant (K_S) describes the overall affinity of an organism for its growth-limiting substrate, while the Michaelis–Menten constant (K_M) can be directly expressed as a ratio of the elementary rate constants acting on the enzyme–substrate complex. In a description of growth under substrate-

limited conditions, it would seem logical to assume that the substrate transporter plays an important role, as this is likely to be the enzyme with which the cell senses the substrate. As such, one could expect the affinity of the cell for the growth-limiting substrate (K_S) to be related to the affinity of the substrate transporter (K_M).

In addition to the Monod equation, several other functional dependencies of μ for a single limiting substrate have been proposed. For a historical review of different models for microbial growth kinetics, see Jannasch & Egli (1993); for a comparison of different growth models, see Button (1993). Many of these models are very similar to the Monod model, but might contain additional parameters, such as the Moser (1958) and Contois (1959) models, while others are fundamentally different, such as the Blackman (1905), Teissier (1936) (also known as the exponential model) and logarithmic models (Westerhoff *et al.*, 1982). While some attempts have been made to give a mechanistic interpretation of the Monod constant, these were never really successful (Liu, 2007).

Systems biology attempts to understand systemic behaviour on the basis of the characteristics of system components. From such a perspective it would be extremely interesting to test whether it is possible to relate a systemic property, such as the K_S of an organism, to the local property of an enzyme (K_M). We have addressed this question using a theoretical and an experimental approach.

In a metabolic control analysis of the problem we could relate the two constants via the growth control of the substrate transporter. In glucose-limited chemostat cultures of the yeast *Saccharomyces cerevisiae* we experimentally determined K_S and K_M , and made the intriguing observation that the overall affinity of the organism is more than a factor of 2 higher than the affinity of the transporter. In addition, from the two constants we could calculate the level of control of μ by the glucose transporter.

METHODS

Strain and cultivation. *Saccharomyces cerevisiae* strain VIN13, kindly provided by the Institute of Wine Biotechnology, Stellenbosch University, South Africa, was used in all the experiments. Cells were grown over a range of dilution rates (D) in glucose-limited chemostat cultures (Bio 110 fermenters; New Brunswick Scientific). The working volume was approximately 650 ml and culture pH was maintained at 5.5 ± 0.1 by automatic addition of 1 M NaOH. The culture was stirred at 250 r.p.m. and sparged with air at a flow rate of 20 l h^{-1} . The temperature was maintained at 30°C . Dissolved oxygen (O_2) was monitored with a DO_2 electrode model InPro6110/160 (New Brunswick Scientific) and kept above 60% saturation.

Medium composition was as described by Verduyn *et al.* (1992) and glucose concentration in the feed was either 1 or 2 mM, ensuring glucose-limiting conditions and a low biomass concentration. No effect of medium glucose concentration on steady-state residual glucose concentration was observed.

For determination of the residual glucose concentration, samples were extracted in an equal volume of cold (4°C) perchloric acid (10%), under pressure to improve the sampling rate. Samples were neutralized using 2 M K_2CO_3 and left on ice for 15 min. To remove precipitated salts and proteins, the samples were centrifuged at $20\,800 \text{ g}$ at 4°C for 10 min. Residual glucose concentrations were determined using the method described by Senn *et al.* (1994).

Cells to be used for glucose uptake experiments were grown in Applikon fermenters with a working volume of 1 l, using the same mineral medium but with a glucose concentration in the medium of 42 mM. The culture was stirred at 750 r.p.m. and sparged with air at a flow rate of 60 l h^{-1} . The culture pH was maintained at 5.0 ± 0.1 .

For zero trans-influx experiments, samples were rapidly taken and washed twice in mineral medium lacking a carbon source using centrifugation at 3000 g and 4°C (SS-34 rotor; Sorvall) for 10 min, and stored on ice for further use. Dry weight was determined by the method of Herbert *et al.* (1971). Metabolite concentrations were determined using HPLC as described in Snoep *et al.* (1990).

Transport assay. Zero trans-influx rates of sugars were determined in a 5 s assay, according to Walsh *et al.* (1994), at 30°C with the modification that growth medium was used instead of phosphate buffer. Cold glucose concentrations were verified by using a glucose oxidase assay: glucose oxidase (Roche Diagnostics), peroxidase and ABTS in 0.5 M Tris/HCl were mixed with standard or sample and incubated at 37°C for 1 h under slow agitation after which the absorbance at 415 nm was measured using a SPECTRAMax PLUS384 Microplate Spectrophotometer (Molecular Devices).

Calculations. All calculations and model simulations were performed with Mathematica version 6 (www.wolfram.com). For the calculation of the internal glucose concentration in steady-state chemostat cultures the following equation was used for the glucokinase reaction (Teusink *et al.*, 2000):

$$v_{\text{GLK}} = \frac{\frac{V_M}{K_{\text{MATP}} K_{\text{MGLC}_i}} \left(\text{GLC}_i \cdot \text{ATP} - \frac{G6P \cdot \text{ADP}}{K_{\text{eq}}} \right)}{\left(1 + \frac{G6P}{K_{\text{MG6P}}} + \frac{\text{GLC}_i}{K_{\text{MGLC}_i}} \right) \cdot \left(1 + \frac{\text{ADP}}{K_{\text{MADP}}} + \frac{\text{ATP}}{K_{\text{MATP}}} \right)} \quad (2)$$

where $K_{\text{eq}}=3800$, $K_{\text{MGLC}_i}=0.08 \text{ mM}$, $K_{\text{MG6P}}=30 \text{ mM}$, $K_{\text{MATP}}=0.15 \text{ mM}$, $K_{\text{MADP}}=0.23 \text{ mM}$, $G6P=2.5 \text{ mM}$, $\text{ATP}=2.5 \text{ mM}$, $\text{ADP}=1.3 \text{ mM}$ as the parameter values and steady-state metabolite concentrations taken from Teusink *et al.* (2000). Throughout the manuscript we will use the symbol K_{MX} for the binding constant of an enzyme for metabolite X, and V_M for the limiting rate of an enzyme. For the V_M of glucokinase, a value of $1779 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$ was used, as determined by Daran Lapujade *et al.* (2007) for aerobic, glucose-limited growth of *S. cerevisiae* at $D=0.1 \text{ h}^{-1}$, which is in good agreement with the $2 \text{ U (mg protein)}^{-1}$ as reported by Postma *et al.* (1989). Using this equation, the internal glucose concentration was determined at a glycolytic flux of 40 and 282 $\text{nmol (mg protein)}^{-1} \text{ min}^{-1}$, as measured in this study at $D=0.1$ and 0.35 h^{-1} , respectively.

For fitting of the kinetic parameters of glucose transport kinetics, the following objective function was minimized:

$$\sum \left(\text{data}(n) - \frac{V_{M1}(\text{GLC}(n) - \text{GLC}_i)}{K_{M1} + \text{GLC}(n) + \text{GLC}_i} + \frac{V_{M2}(\text{GLC}(n) - \text{GLC}_i)}{K_{M2} + \text{GLC}(n) + \text{GLC}_i} \right)^2 + w \cdot \left(J_{\text{GLC}} - \frac{V_{M1}(\text{GLC}_c - \text{GLC}_{ic})}{K_{M1} + \text{GLC}_c + \text{GLC}_{ic}} + \frac{V_{M2}(\text{GLC}_c - \text{GLC}_{ic})}{K_{M2} + \text{GLC}_c + \text{GLC}_{ic}} \right)^2 \quad (3)$$

where subscripts 1 and 2 refer to the low and high transport components, respectively, $\text{data}(n)$ is the transport activity data for n different glucose concentrations $[\text{GLC}(n)]$, GLC_i is the internal glucose concentration during the transport assay, J_{GLC} is the steady-state glucose uptake rate in the chemostat, GLC_c is the residual glucose concentration in the chemostat, GLC_{ic} is the steady-state internal glucose concentration in the chemostat, and w is a factor to give more weight to the steady-state chemostat uptake rate, which is a single mean value, as opposed to the large number of kinetic data points (a value of 5 was used for w ; increasing to higher values did not significantly influence the outcome of the optimization).

Core model for chemostat system. The chemostat is an ideal instrument for studying the relationship between μ and the concentration of a growth-limiting substrate (S).

In a chemostat experiment the medium is composed such that one of the substrates will be growth-rate-limiting. This makes it possible to set μ via the influx rate of the medium. At steady-state, the $\mu=D$, defined as the medium flow rate/culture volume. As such, it is possible to construct a plot of μ against S by varying D and measuring the steady-state residual substrate concentration.

The Monod equation (equation 1) can be fitted to such a dataset, to estimate the maximal specific growth rate μ_{max} and Monod constant K_S .

To understand how a change in medium influx rate ultimately leads to a change in μ , it is useful to first analyse a minimal chemostat system, consisting of four variables: S , S_{in} , X and B (Fig. 1).

Such a system can be described by the following set of differential equations, with D reflecting the pump reactions:

$$\frac{dS}{dt} = D \cdot (S_f - S) - v_{\text{tr}} \quad (4)$$

$$\frac{dS_{\text{in}}}{dt} = v_{\text{tr}} - v_{\text{in}} \quad (5)$$

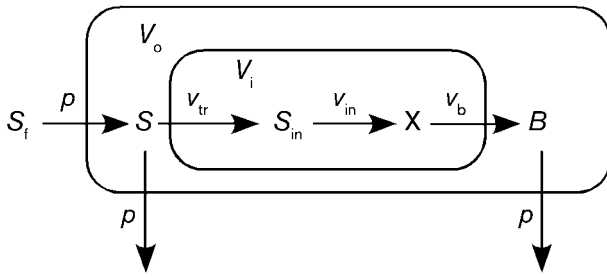


Fig. 1. Scheme for the simple chemostat set-up as used in the theory and model sections. The chemostat consists of two external variables (in extracellular volume V_o): the growth-limiting substrate concentration (S) and the biomass concentration (B). The biological system consists of two variables (in intracellular volume V_i), S_{in} , the internal substrate concentration, and X , a precursor for biomass formation. The micro-organism consists of three enzyme-catalysed reactions: v_{tr} , the substrate transporter, v_{in} , an internal reaction producing X , and v_b , a biosynthetic reaction forming biomass from X . The chemostat instrument consists of pump reactions (p) leading to the influx of substrate and efflux of effluent from the culture (containing substrate and biomass).

$$\frac{dX}{dt} = v_{in} - v_b \quad (6)$$

$$\frac{dB}{dt} = v_b - D \cdot B \quad (7)$$

Two core models with different kinetic types for the substrate transporter were constructed. The parameter values for the models vary, and were chosen such that the steady-state behaviour of the models follows Monod kinetics. The models can be accessed from the JWS Online database (Olivier & Snoep, 2004) at <http://jij.biochem.sun.ac.za/database/snoep1/> and <http://jij.biochem.sun.ac.za/database/snoep2/>, respectively

RESULTS

Theory

For this analysis we have used the metabolic control analysis framework originally developed by Kacser & Burns (1973) and Heinrich & Rapoport (1974), which has been expanded extensively since then (for example, see Fell, 1992, 1996; Heinrich & Schuster, 1996) and has also been applied to chemostat cultures (Small, 1994; Snoep *et al.*, 1994). Any effect of the pump rate on the μ must have been mediated via either of the two external variables, S or B :

$$G_p^\mu = G_p^S \cdot R_S^\mu + G_p^B \cdot R_B^\mu \quad (8)$$

$$= G_p^S \cdot \varepsilon_S^{v_{tr}} \cdot C_{v_{tr}}^\mu + G_p^B (\varepsilon_B^{v_{tr}} \cdot C_{v_{tr}}^\mu + \varepsilon_B^{v_b} \cdot C_{v_b}^\mu) \quad (9)$$

where G_p^μ is a global control coefficient, defined as $\frac{d\mu}{dp} \cdot \frac{p}{\mu}$, and describes the percentage change in μ upon a 1 percentage change in pump rate p . Since μ is proportional to D (and thus also to p), $G_p^\mu = 1$. The change in steady-state concentrations of S and B by p is quantified by G_p^S and G_p^B ,

respectively, and their effect on the local response of the micro-organism is given by R_S^μ and R_B^μ , respectively. These local responses are defined at the steady-state concentration of S and B and can be interpreted as the change in μ of the isolated micro-organism upon a change in S or B (e.g. $R_S^\mu = \frac{d\mu}{dS} \cdot \frac{S}{\mu} \Big|_{S^*, B^*}$, where $*$ denotes the steady-state concentrations of the respective variables). Assuming that S is only sensed by its transporter tr , the effect of a change in S is mediated via its effect on the transporter activity v_{tr} , quantified by its elasticity coefficient ($\varepsilon_S^{v_{tr}} = \frac{\partial v_{tr}}{\partial S} \cdot \frac{S}{v_{tr}}$). The resulting effect of the change in transport activity on steady-state is given by the control coefficient of the transporter on μ ($C_{v_{tr}}^\mu = \frac{d\mu}{dv_{tr}} \cdot \frac{v_{tr}}{\mu} \Big|_{S^*, B^*}$). In a similar way, equation 9 describes the effects of p on biomass B , where both reactions v_{tr} and v_b are sensitive to changes in the biomass concentration.

The steady-state biomass concentration is equal to the biomass yield on substrate Y_S^B times the substrate that is consumed by the organisms: $B = Y_S^B \cdot (S_f - S)$, where S_f is the substrate concentration in the feed. Assuming that Y_S^B is relatively constant and that the growth limiting substrate concentration will generally be low and insignificant compared to S_f (at low specific growth rates), we can derive:

$$G_p^B = \frac{dB}{dp} \cdot \frac{p}{B} = \frac{d(Y_S^B(S_f - S))}{dp} \cdot \frac{p}{Y_S^B(S_f - S)} \quad (10)$$

$$= \frac{-dS}{dp} \cdot \frac{p}{S_f - S} \quad (11)$$

$$= G_p^S \cdot \frac{S}{S_f - S} \approx 0 \quad (12)$$

Thus, at low specific growth rates, changing the pump rate does not affect the biomass concentration. Then, equation 9 reduces significantly, and substituting $G_p^\mu = 1$ leads to:

$$G_p^S = \frac{1}{\varepsilon_S^{v_{tr}} \cdot C_{v_{tr}}^\mu} \equiv \frac{1}{R_S^\mu} \quad (13)$$

Equation 13 relates the change in substrate concentration after changing the pump rate to the local elasticity and control coefficients of the substrate transporter. This equation is general, i.e. not restricted to a specific growth rate function or transport rate equation, and holds if the residual substrate concentration is much lower than the medium substrate concentration, i.e. at low dilution rates.

From the Monod equation, the response coefficient of μ with respect to S can be derived:

$$R_S^\mu = \frac{d\mu}{dS} \cdot \frac{S}{\mu} = \frac{K_S}{S + K_S} \quad (14)$$

If we substitute a specific rate equation for the substrate transporter, we can derive a relationship between the Monod constant K_S and the Michaelis–Menten constant

K_M . For instance, in the case of Michaelis–Menten kinetics for the transporter:

$$v_{tr} = \frac{V_M \cdot S}{K_M + S} \quad (15)$$

its elasticity equals:

$$\varepsilon_S^{v_{tr}} = \frac{\partial v_{tr}}{\partial S} \cdot \frac{S}{v_{tr}} = \frac{K_M}{K_M + S} \quad (16)$$

Substituting equation 16 and equation 14 into equation 13 and rearranging:

$$C_{v_{tr}}^\mu = \frac{1 + \frac{S}{K_M}}{1 + \frac{S}{K_S}} \quad (17)$$

When Michaelis–Menten kinetics (equation 15) are used to describe the transport step for the growth-limiting substrate, this step will have full control on growth rate, i.e. $C_{v_{tr}}^\mu = 1$, and from equation 17 it follows that in that case $K_M = K_S$.

The Michaelis–Menten equation is well known and widely used in studies on isolated enzymes, but its applicability for the analysis of systems of linked reactions is very restricted, as it assumes the absence of product. However, in a system of linked reactions, product concentrations can never be zero, as they function as substrates for the next reaction. Thus, the effect of product on enzyme activity must be taken into account. This effect can be of a kinetic (competition with the substrate for binding to the active site) or a thermodynamic type (with increasing product concentration the Gibbs free energy of a reaction becomes less negative).

If we first consider the kinetic effect of product on the enzyme activity, equation 15 can be extended to:

$$v_{tr} = \frac{V_M \cdot \frac{S}{K_{MS}}}{1 + \frac{S}{K_{MS}} + \frac{P}{K_{MP}}} \quad (18)$$

and its elasticity equals:

$$\varepsilon_S^{v_{tr}} = \frac{K_{MS} \left(1 + \frac{P}{K_{MP}} \right)}{K_{MS} \left(1 + \frac{P}{K_{MP}} \right) + S} \quad (19)$$

Thus, if one would assay the sensitivity of the transporter for its substrate, while keeping the internal product concentration constant at the steady-state concentration, equation 19 is equivalent to equation 15 with $K'_{MS} = K_{MS} \left(1 + \frac{P}{K_{MP}} \right)$, and equation 17 holds, with K'_{MS} replacing K_M .

An example of such a system could be the PTS glucose transport system in *Escherichia coli* which is an active transport system, effectively irreversible but still product sensitive.

For a reversible reaction, e.g. assuming a symmetrical transporter for the facilitated transport of glucose in yeast, the following rate equation can be used:

$$v_{tr} = \frac{\frac{V_M}{K_M} (S - S_{in})}{1 + \frac{S}{K_M} + \frac{S_{in}}{K_M}} \quad (20)$$

and its elasticity equals:

$$\varepsilon_S^{v_{tr}} = \frac{S}{S - S_{in}} \cdot \frac{K_M + 2 \cdot S_{in}}{K_M + S + S_{in}} \quad (21)$$

When equation 21 (instead of equation 16) is substituted in equation 13, it is not possible to express K_S in terms of K_M without knowing the internal glucose concentration. If we consider the two extreme cases, $S_{in} = 0$ and $S_{in} = S$, it can be shown that in the latter case there is no relation between K_S and K_M , and the elasticity of the transporter approaches infinity, while in the first case equation 21 reduces to equation 16 (as does equation 19).

Importantly, when using product-sensitive rate equations such as equations 18 and 20, the transporter will not automatically have a control on growth rate of 1. More specifically, unless the transporter is completely limiting growth, its flux control coefficient will be less than 1. In cases where the transporter is far away from equilibrium (as expected for the uptake of the growth-limiting component), it can then be derived that K_S will be smaller than K_M . This is a rather counter-intuitive result; the affinity of an organism for its growth-limiting substrate is greater than the affinity of the enzyme with which the organism senses (and transports) the substrate.

Core models

To illustrate the theoretical findings, core models simulating different transport kinetics were constructed. Each of the core models has the same network structure (as shown in Fig. 1), but uses a different kinetic type for the transporter, with an irreversible but product-sensitive transporter in core model 1, and a reversible symmetrical transporter in core model 2. The parameter values for both core models were chosen such that the culture can be described by Monod kinetics (Table 1), and the steady-state results for the models are shown in Table 2. For the determination of μ_{max} and K_S , a range of D values from 0.01 to 0.45 h^{-1} was analysed for steady-state, while the other model results listed in Table 2 were obtained at $D = 0.1 h^{-1}$. For the irreversible transporter, an elasticity coefficient close to 1 was calculated (equation 19), which one would expect at the low residual substrate concentration, i.e. the rate is proportional to the substrate concentration. For the reversible transporter as used in core model 2, a much higher elasticity was calculated using equation 21, which can be understood on the basis of thermodynamic back-pressure. For both models, the effect of the pump on the residual substrate concentration (C_p^S) is comparable. From the $\varepsilon_S^{v_{tr}}$ and C_p^S values, the control of the

Table 1. Parameter values for the chemostat core models

Note that the internal reactions, i.e. v_{tr} , v_{in} and v_b , are multiplied by B as their rates are proportional to the biomass in the culture. In addition, the internal volume was modelled as constant ($V_i=V_o=0.0001$) for reasons of simplicity. The medium substrate concentration, S_b , was chosen to be 20 mM. D was scanned from 0.01 to 0.45 h^{-1} .

Reaction	Equation	Parameters			
		V_M (mM h^{-1})	K_{MS} (mM)	K_{MP} (mM)	K_{eq}
Core model 1					
v_{tr}	18	9	0.1	0.1	
v_{in}	20	10	1	1	100
v_b	15	0.52	2		
Core model 2					
v_{tr}	20	9	0.9	0.9	1
v_{in}	20	10	1	1	100
v_b	15	0.52	2		

transporter on μ , C_{vtr}^μ , can be calculated using equation 13 to be 0.82 and 0.28, respectively, for core models 1 and 2. The same values for C_{vtr}^μ were also obtained directly when the ‘bacterium’ in the core model was analysed at steady-state values of S and B , i.e. when analysing the system of v_{tr} , v_{in} and v_b at clamped concentrations of S and B .

Importantly, according to our derivations it should be possible from the K_S , K'_M , S and S_{in} values to estimate C_{vtr}^μ using equation 17. Indeed for core model 1, the equation gave a good estimate for the control of the transporter on μ ($(1 + 0.00134/(0.1 + 0.0197))/(1 + 0.00134/0.0054) = 0.81$). For the model with the reversible transport kinetics, we have derived that the control of the transporter on μ can be calculated from the K_M , K_S , S and S_{in} values using equations 21, 14 and 13. Indeed, using these equations a value of 0.28 was obtained for C_{vtr}^μ , again close to the result obtained with the model simulations. These core models only serve to illustrate the theoretical analysis section with numerical examples; the parameter values are not experimentally determined and should not be seen as realistic values.

Experimental results

The Monod equation describes how μ of an organism is dependent on the concentration of a single substrate. We

have used chemostat cultures to achieve a single substrate limitation while having control over μ via setting of the dilution rate. At steady-state $\mu = D$, and by determining the steady-state residual substrate concentration at various D values, the relationship between the limiting substrate concentration and μ can be studied.

Measurement of the growth-limiting substrate concentration is usually difficult because the concentrations are low and likely to change during sampling unless a method is used that rapidly stops metabolism. For these reasons we used glucose-limited chemostat cultures of the yeast *Saccharomyces cerevisiae*, which is known to have a low affinity for this substrate, and we operated the chemostat at low culture densities and stopped metabolism by rapid sampling in acid.

S. cerevisiae VIN13 was grown at a range of D values between 0.05 and 0.48 h^{-1} , and μ as a function of the residual glucose concentration is plotted in Fig. 2. Fitting the Monod equation on the dataset yields a K_S estimate of 0.12 mM (asymptotic SEM 0.009) and a μ_{max} value of 0.50 h^{-1} (asymptotic SEM 0.011).

In the chemostat, the steady-state glycolytic flux (q_{glc}) can be calculated from the medium glucose concentration (GLC_f), the residual glucose concentration (GLC), the dilution rate (D) and the biomass concentration, using $q_{glc} = \frac{GLC_f - GLC}{Biomass} \cdot D$. Steady-state uptake rates of glucose

in the chemostat were determined over the dilution range 0.05–0.48 h^{-1} (Fig. 3). At low D values, a linear relationship was observed between q_{glc} and D , indicating that $Y_{GLC}^{Biomass}$ is constant. In addition, up to $D=0.15$ h^{-1} the residual glucose concentration is <5% of the medium glucose concentration, and no significant change in biomass concentration was observed between $D=0.05$ and 0.15 h^{-1} (in agreement with the assumptions on the basis of which equation 12 was derived).

At D values higher than 0.4 h^{-1} , a dramatic increase in the slope of q_{glc} against D is observed (Fig. 3), indicating a decrease in $Y_{GLC}^{Biomass}$. This phenomenon is well known as the Crabtree effect, and shows the shift from purely oxidative metabolism to fermentative metabolism with the production of ethanol.

To be used as a growth substrate, glucose first needs to be taken up, for which yeast is known to have several transporters. To investigate the relationship between the affinity of the glucose transport step (K_M) and the overall

Table 2. Steady-state results obtained with the core models

Model	Monod kinetics		Steady-state			Control properties	
	μ_{max} (h^{-1})	K_S (mM)	S (mM)	S_{in} (mM)	B (mM)	ϵ_S^{vtr}	G_p^S
Core 1	0.50	0.0054	0.0013	0.0197	19.998	0.989	1.23
Core 2	0.51	0.126	0.030	0.0197	19.970	2.84	1.265

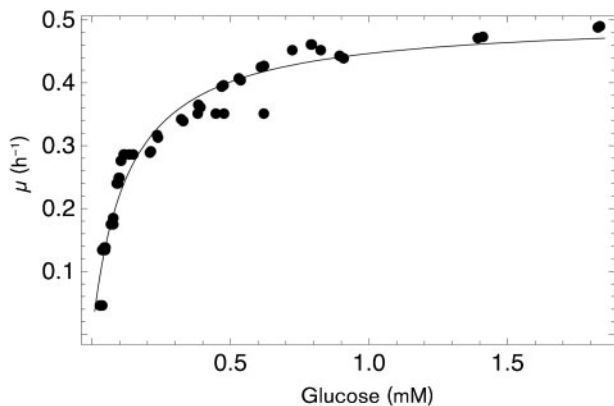


Fig. 2. μ as a function of the residual substrate concentration. *S. cerevisiae* was grown over a range of D values and steady-state residual glucose concentrations were determined. The Monod curve is fitted through the data points.

affinity of the cell for glucose (K_S), we harvested yeast cells at two D values and characterized the glucose transport step.

In Fig. 4 the glucose uptake kinetics for yeast grown at a D of 0.1 h^{-1} are shown in an Eadie–Hofstee plot, where the data were fitted, assuming zero trans-influx kinetics, to a two-component transport system, consisting of a high-affinity [$K_{M1} 0.75 \text{ mM}$, $v_{M1} 506 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$] and a low-affinity transporter [$K_{M2} 201 \text{ mM}$, $v_{M2} 787 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$]. For cells cultivated at $D=0.35 \text{ h}^{-1}$, similar results were obtained [$K_{M1} 0.72 \text{ mM}$, $v_{M1} 632 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$; $K_{M2} 93 \text{ mM}$, $v_{M2} 569 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$]. These results indicate

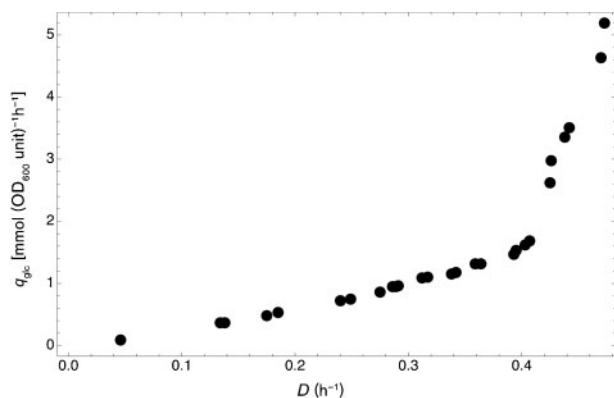


Fig. 3. Steady-state glycolytic flux as a function of D . *S. cerevisiae* was grown in glucose-limited chemostat cultures over a range of D values, and the steady-state uptake rate of glucose was determined. The uptake rate is expressed per OD_{600} unit; due to the low densities at which the cells were grown it was not possible to get an accurate dry weight measurement.

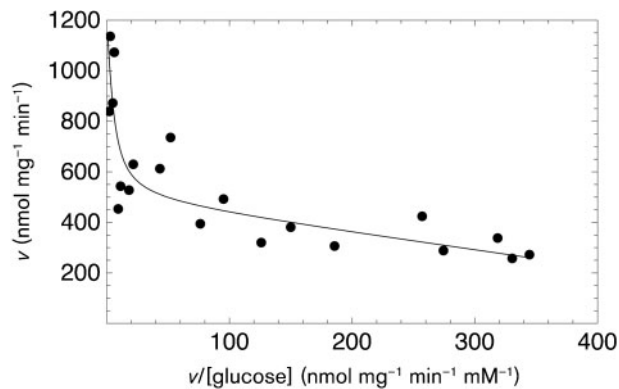


Fig. 4. Eadie–Hofstee plot for glucose transport kinetics. Transport rates were determined using ^{14}C -labelled glucose in zero trans-influx assays for *S. cerevisiae*, grown in glucose-limited chemostat cultures at $D=0.1 \text{ h}^{-1}$. The line shows the best fit to a two-component transport system.

that there was not a big effect of the D on the high-affinity system for glucose transport.

The steady-state uptake rate of glucose in the chemostat equals 40 and $282 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$ at $D=0.1$ and 0.35 h^{-1} , respectively (assuming 50% of the dry weight is protein). With the very low residual glucose concentration in the chemostat (0.03 and 0.29 mM at $D=0.1$ and 0.35 h^{-1} , respectively) the measured uptake kinetics cannot explain the measured steady-state glucose uptake rates, even if the internal glucose concentration was zero. For the chemostat-grown cells, the internal glucose concentration cannot be zero, otherwise there would be no glycolytic flux. An estimate for the internal glucose concentration can be made on the basis of the steady-state flux and the kinetics of the glucokinase. Using the kinetic information on the glucokinase, and the steady-state metabolite concentrations from the kinetic model as published by Teusink *et al.* (2000) together with the expression level as determined by Daran-Lapujade *et al.* (2007) for glucose-limited chemostat culture at $D=0.1 \text{ h}^{-1}$, an internal glucose concentration of 0.003 mM was calculated, which is close to the $1 \mu\text{M}$ concentration suggested by Postma *et al.* (1989), (for $D=0.35 \text{ h}^{-1}$ an internal glucose concentration of 0.025 mM was calculated; see Methods for details).

If this non-zero glucose concentration is taken into account for the predicted glucose uptake rate on the basis of the measured *in vitro* kinetics, then an even greater difference between measured and predicted glucose uptake rate is observed.

The most likely explanation for this discrepancy is that the internal glucose concentration in the *in vitro* uptake experiments is not negligible and leads to an overestimation of the K_M for glucose. An objective function that sums the squared differences between the experimental data and

the model prediction was constructed (allowing for a non-zero internal glucose concentration in the glucose assay and adding, as an additional constraint, the glucose uptake rate in the chemostat; see Methods for details). Minimizing the objective function resulted in the following parameter values for the cells grown at $D=0.1 \text{ h}^{-1}$: for the high-affinity transporter, $K_M=0.30 \text{ mM}$ and $v_{\max}=494 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$, and for the low-affinity system, $K_M=187 \text{ mM}$ and $v_{\max}=786 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$, with an internal glucose concentration in the *in vitro* assay of 0.15 mM . At a dilution rate of 0.35 h^{-1} an identical internal glucose concentration was obtained with the following kinetic parameters: $K_{M1}=0.27 \text{ mM}$, $K_{M2}=87 \text{ mM}$, $v_{\max1}=619 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$, $v_{\max2}=578 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$.

Applying equations 13 and 21, a response coefficient of yeast for glucose of 0.8 and an elasticity of the transporter for glucose of 1.02 can be calculated at $D=0.1 \text{ h}^{-1}$; at $D=0.35 \text{ h}^{-1}$ response and elasticity coefficients of 0.29 and 0.60, respectively, were obtained. From these, using equation 13, a control of the transporter on the specific growth rate of 0.78 and 0.49 can be calculated at $D=0.1$ and 0.35 h^{-1} respectively. This means that at a low external glucose concentration of 0.03 mM , the glucose transporter has 78% of growth rate control (note that this is the control on growth rate for a bacterium that is isolated from the chemostat with the external metabolites clamped at steady-state values). Similarly, a control rate of 49% could be calculated at an external glucose concentration of 0.29 mM .

DISCUSSION

Growth and reproduction are essential functions of living organisms. In growing cells, thousands of reactions are coordinated to build all cellular components necessary for the production of daughter cells. Despite its importance in many diseases (e.g. cancer), control and (dys-)regulation of growth rate is poorly understood, i.e. it is not known to what extent the different reactions in the metabolic network limit μ .

The dependency of growth rate on external parameters is much better studied, especially for unicellular organisms limited in growth by the availability of a single substrate (S), where typically a hyperbolic relationship is observed between μ and S. We focused on the description of that hyperbolic relationship using the Monod equation (equation 1), which fitted our data well. Intriguingly, the Monod equation, describing the co-ordinated response of thousands of reactions, is identical to the Michaelis–Menten equation, describing the activity of an isolated enzyme, and although Monod has stated that K_S is not equal to K_M , he did suggest that there may be some relationship between the two parameters (Monod, 1949). In the chemostat, the pump rate controls μ via its effect on the growth-limiting substrate concentration, G_p^S . This control can be related to

the elasticity for glucose and the control on growth rate of the substrate transporter via equation 13. This equation is general, but when a specific growth model, such as the Monod model, and a kinetic type for the transporter are inserted, it is possible to relate the K_M of the transporter to the K_S of the organism. An important constraint for the relationship is that it is dependent on whether the elasticity of the transporter can be expressed as a function of its K_M . For irreversible enzymes, this is always possible (although the value may also be dependent on the internal substrate concentration), but for reversible enzymes close to equilibrium the importance of the K_M value for the elasticity coefficient becomes small. Then it will, in general, not be possible to relate the K_M of the transporter to the K_S of the organism. In addition, under such conditions the control of the transporter on μ will be very small.

An important finding of our study is that from the relationship between K_S and K_M it is possible to calculate the control of the transporter on μ if the residual substrate concentration is known (in case of reversible kinetics the internal concentration needs to be known if it is not negligible). Thus for *S. cerevisiae* it was estimated that the transporter has 78% of growth control at a clamped glucose concentration of 0.03 mM . The experimental approach we followed is similar to the one of Postma *et al.* (1989) where a K_S of $0.11\text{--}0.39 \text{ mM}$ was determined (although the data could not be described very well by Monod kinetics) with a high-affinity K_M of 1 mM . In addition, a residual glucose concentration at $D=0.1 \text{ h}^{-1}$ of 0.11 mM was measured and an internal glucose concentration of 0.001 mM was calculated (Postma *et al.*, 1989). Using our theoretical analysis, it can be calculated from these data that the glucose transporter controls μ at 55–85% (dependent on the K_S value).

The analysis we have given is general and can be applied to experimental data of other organisms as well. Good experimental data are available for another model organism, *E. coli*, for which a K_S value of $0.41 \text{ }\mu\text{M}$ was obtained with a residual glucose concentration of $0.20 \text{ }\mu\text{M}$ at $D=0.3 \text{ h}^{-1}$ (Senn *et al.*, 1994). From these data a response coefficient of glucose for $\mu=0.67$ can be calculated using equation 14. A K_M value for glucose in the PTS of $20 \text{ }\mu\text{M}$ has been reported (Stock *et al.*, 1982). In a first assumption to estimate the elasticity of the PTS for glucose, a random order rapid equilibrium type of kinetics was used for the overall reaction catalysed by the PTS. The elasticity for such a rate equation is equal to the one in equation 18. From the elementary rate constants given in Rohwer *et al.* (2000), it can be calculated that the effect of the internal glucose 6-phosphate concentration is negligible due to the low affinity of the enzyme for this product. Thus an elasticity of the PTS for glucose of 0.99 can be calculated at an external glucose concentration of $0.2 \text{ }\mu\text{M}$. The elasticity of the PTS system for glucose can also be estimated directly from the detailed kinetic model (Rohwer *et al.*, 2000) as 0.97. These data indicate that in *E. coli* the glucose transporter controls μ at 67% (or 69% if an elasticity of 0.97 is used) at the low

glucose concentrations obtained in glucose-limited chemostat culture at $D=0.3 \text{ h}^{-1}$.

In this study we have shown that the Monod constant, although in itself purely empirical, can be related to the Michaelis–Menten constant of the transporter for the growth-limiting substrate. Key to the analysis is to use the Monod equation to express the sensitivity of μ in terms of the Monod constant. Subsequently, this sensitivity was related to the elasticity of the transporter for the substrate and the control of the transporter on μ . Thus K_S and K_M values can be related to each other via the control of the transporter on μ . This leads to the interesting result that this control, which is often difficult to measure directly, can be estimated from the Monod and Michaelis–Menten constants, as we have shown for *S. cerevisiae* and *E. coli*.

From a systems biology perspective, these results have a wider implication than just finding the relationship between a phenomenological and a mechanistic constant. For a field that has as one of its aims the understanding of systemic behaviour on the basis of the characteristics of its components (Snoep & Westerhoff, 2005), these results have far-reaching implications in that it is possible to express a systemic property, describing the overall sensitivity of an organism for its growth-limiting substrate, as a function of the characteristics of the transporter for the substrate. Crucial in the analysis was to link the importance of the component, as expressed by its growth control, to its sensitivity for the growth-limiting substrate. Under the specific conditions as prevail in glucose-limited chemostat cultures, the glucose transport step holds more than 50% of growth rate control, which is quite amazing when one considers that growth is a concerted action of several thousands of enzymes. The interplay between a good theoretical framework such as metabolic control analysis, modelling and experiment, as used in this study, is illustrative of the type of approaches that we think will be essential for addressing systems biology problems.

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REFERENCES

- Blackman, F. F. (1905).** Optima and limiting factors. *Ann Bot (Lond)* **19**, 281–295.
- Button, D. K. (1993).** Nutrient-limited microbial growth kinetics: overview and recent advances. *Antonie Van Leeuwenhoek* **63**, 225–235.
- Contois, D. E. (1959).** Kinetics of bacterial growth: relationship between population density and specific growth rate of continuous culture. *J Gen Microbiol* **21**, 40–50.
- Daran-Lapujade, P., Rossell, S., van Gulik, W. M., Luttkik, M. A. H., de Groot, M. J. L., Slijper, M., Heck, A. J. R., Daran, J.-M., de Winde, J. H. & other authors (2007).** The fluxes through glycolytic enzymes in *Saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels. *Proc Natl Acad Sci U S A* **104**, 15753–15758.
- Fell, D. A. (1992).** Metabolic control analysis: a survey of its theoretical and experimental development. *Biochem J* **286**, 313–330.
- Fell, D. (1996).** *Understanding the Control of Metabolism*. Portland Press.
- Heinrich, R. & Rapoport, T. A. (1974).** A linear steady-state treatment of enzymatic chains: general properties, control and effector strength. *Eur J Biochem* **42**, 89–95.
- Heinrich, R. & Schuster, S. (1996).** *The Regulation of Cellular Systems*. Chapman and Hall.
- Herbert, D., Phipps, P. J. & Strange, R. E. (1971).** Chemical analysis of microbial cells. *Methods Microbiol* **5B**, 209–344.
- Jannasch, H. W. & Egli, T. (1993).** Microbial growth kinetics: a historical perspective. *Antonie Van Leeuwenhoek* **63**, 213–224.
- Kacser, H. & Burns, J. A. (1973).** The control of flux. *Symp Soc Exp Biol* **27**, 65–104.
- Liu, Y. (2007).** Overview of some theoretical approaches for derivation of the Monod equation. *Appl Microbiol Biotechnol* **73**, 1241–1250.
- Monod, J. (1949).** The growth of bacterial cultures. *Annu Rev Microbiol* **3**, 371–394.
- Moser, A. (1958).** *The Dynamics of Bacterial Populations Maintained in the Chemostat*, Publication 614. Washington, DC: The Carnegie Institution.
- Olivier, B. G. & Snoep, J. L. (2004).** Web-based kinetic modelling using JWS Online. *Bioinformatics* **20**, 2143–2144.
- Postma, E., Scheffers, W. A. & Van Dijken, J. P. (1989).** Kinetics of growth and glucose transport in glucose-limited chemostat cultures of *Saccharomyces cerevisiae* cbs 8066. *Yeast* **5**, 159–165.
- Rohwer, J. M., Meadow, N. D., Roseman, S., Westerhoff, H. V. & Postma, P. W. (2000).** Understanding glucose transport by the bacterial phospho-enolpyruvate:glucose phosphotransferase system on the basis of kinetic measurements *in vitro*. *J Biol Chem* **275**, 34909–34921.
- Senn, H., Lendenmann, U., Snozzi, M., Hamer, G. & Egli, T. (1994).** The growth of *Escherichia coli* in glucose-limited chemostat cultures: a reexamination of the kinetics. *Biochim Biophys Acta* **1201**, 424–436.
- Small, J. R. (1994).** Design and analysis of chemostat experiments using Metabolic Control Analysis: a top-down approach. *Microbiology* **140**, 2439–2449.
- Snoep, J. L. & Westerhoff, H. V. (2005).** From isolation to integration, a systems biology approach for building the silicon cell. In *Systems Biology: Definitions and Perspectives*. Edited by L. Alberghina & H. Westerhoff. New York: Springer.
- Snoep, J. L., Teixeira de Mattos, M. J., Postma, P. W. & Neijssel, O. M. (1990).** Involvement of pyruvate dehydrogenase in product formation in pyruvate-limited anaerobic chemostat cultures of *Enterococcus faecalis* NCTC 775. *Arch Microbiol* **154**, 50–55.
- Snoep, J. L., Jensen, P. R., Groeneveld, P., Molenaar, D., Kholodenko, B. N. & Westerhoff, H. V. (1994).** How to determine control of growth rate in the chemostat, using metabolic control analysis to resolve the paradox. *Biochem Mol Biol Int* **33**, 1023–1032.
- Stock, J. B., Waygood, E. B., Meadow, N. D., Postman, P. W. & Roseman, S. (1982).** Sugar transport by the bacterial phosphotransferase system. *J Biol Chem* **257**, 14543–14552.
- Teissier, G. (1936).** Les lois quantitatives de la croissance. *Ann Physiol Physicochim Biol* **12**, 527–573.

Teusink, B., Passarge, J., Reijenga, C. A., Esgalhad, E., Van der Weijden, C. C., Schepper, M., Walsh, M. C., Bakker, B. M., Van Dam, K. & other authors (2000). Can yeast glycolysis be understood in terms of *in vitro* kinetics of the constituent enzymes? Testing biochemistry. *Eur J Biochem* **267**, 5313–5329.

Verduyn, C., Postma, E., Scheffers, W. A. & van Dijken, J. P. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**, 501–517.

Walsh, M. C., Smits, H. P., Scholte, M. & van Dam, K. (1994). Affinity of glucose transport in *Saccharomyces cerevisiae* is modulated during growth on glucose. *J Bacteriol* **176**, 953–958.

Westerhoff, H. V., Lolkema, J. S., Otto, R. & Hellingwerf, K. J. (1982). Thermodynamics of growth. Non-equilibrium thermodynamics of bacterial growth. The phenomenological and the mosaic approach. *Biochim Biophys Acta* **683**, 181–220.

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