Approaches to Biosimulation of Cellular Processes

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Abstract Modelling and simulation are at the heart of the rapidly developing field of *systems biology*. This paper reviews various types of models, simulation methods, and theoretical approaches that are presently being used in the quantitative description of cellular processes. We first describe how molecular interaction networks can be represented by means of stoichiometric, topological and kinetic models. We briefly discuss the formulation of kinetic models using mesoscopic (stochastic) or macroscopic (continuous) approaches, and we go on to describe how detailed models of molecular interaction networks (silicon cells) can be constructed on the basis of experimentally determined kinetic parameters for cellular processes. We show how theory can help in analyzing models by applying control analysis to a recently published silicon cell model. Finally, we review some of the theoretical approaches available to analyse kinetic models and experimental data, respectively.

Key words systems biology · biosimulations · networks · mathematical models · kinetic models

Introduction—Why Model?

The process of modelling is intrinsic to all scientific activities, provided that one considers such activities as going beyond the plain description of the observed phenomena. The purpose of model building is, therefore, intimately related to the purpose of doing science,

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i.e., to the acquisition of useful knowledge and the development of a deeper understanding of the underlying mechanisms and processes. Modelling and simulation are effective tools to support the development of such understanding. However, the structural and functional complexity of living systems is much greater than anything we can describe in terms of available mathematical concepts or computational tools. Biological models are therefore always abstractions, useful to the extent that they can provide us with new insight and knowledge.

Cell biology deals with a wide variety of different problems, including metabolic and electrophysiological processes, cell-to-cell communication, immunological reactions, and cell division processes. Several of these phenomena have been considered in other contributions to the present issue [1–4]. The models that we focus on the present discussion deal with the organization and functioning of intracellular networks composed of interacting molecules. Examples of such networks are metabolic, signalling and genetic networks.

Topological Modelling in Systems Biology

There are multiple ways of modelling intracellular molecular networks. Consider, for instance, a metabolic pathway. The simplest model is a pathway diagram as displayed in standard biochemistry textbooks. In such diagrams all the reactions are depicted as directed edges (arrows) interconnected by nodes representing substrates and products. This kind of model can be straightforwardly transformed into a mathematical description, a so-called *stoichiometric matrix* [5]. Often this matrix is denoted by $\mathbf{N}$. Its rows represent the variable intermediates in the network (m in total), and its columns represent the reactions in the network (r in total). An entry of $\mathbf{N}$ in its i th row and j th column, denoted by $\{\mathbf{N}\}_{ij}$, corresponds to a stoichiometric coefficient. This coefficient represents the number of moles of compound i consumed (if $\{\mathbf{N}\}_{ij} < 0$), or produced (if $\{\mathbf{N}\}_{ij} > 0$), in the j th reaction of the network. This matrix is amenable to many forms of mathematical operation; mostly deriving from linear algebra. This kind of description allows for a purely *stoichiometric analysis* of intracellular molecular networks. Today, stoichiometric models are often used for the analysis of genome-scale metabolic systems that have resulted from whole-genome functional-annotation studies [6, 7].

A signalling network or genetic circuit can be characterized by a so-called *graph*. (Metabolic networks can also be represented by graphs, but this description is used much less frequently than their description in terms of stoichiometric matrices; for exceptions see [8, 9]. Graphs can be constructed in various ways. The simplest way to construct such a graph involves the use of an *adjacency matrix*. Such matrices can be analysed using methods from graph theory (e.g., [10–12]). The adjacency matrix is a square matrix $\mathbf{A}$ where the columns and rows correspond to the identity of the intermediates in the network. $\{\mathbf{A}\}_{ij}$ then denotes the presence of an interaction between the i th and j th intermediate of the network by a 1 and its absence by a 0. The graph can also be drawn by depicting the intermediates in the network as nodes connected by edges denoting a direct interaction. If none of the interactions are considered to have a direction, the matrix $\mathbf{A}$ is symmetrical: $\{\mathbf{A}\}_{ij} = \{\mathbf{A}\}_{ji}$. More often the network is considered to be composed of interactions that do have a direction; meaning that the interaction $a \rightarrow b$ between molecules a and b is considered to be different from $b \rightarrow a$. Then the matrix $\mathbf{A}$ is no longer symmetrical but corresponds to the adjacency matrix of a network resembling a *directed graph*. Analyses based on adjacency matrices could be termed *topological analyses*. This level of description

is further removed from the molecular mechanisms by which these interactions occur than the stoichiometric matrix; it is a more coarse-grained description of the same network.

On a more detailed level of description, a signalling network or genetic circuit can also be described by a stoichiometric matrix (e.g., [13, 14]). But most often we do not yet know all the elementary reactions constituting any given signalling network or genetic circuit. In those cases, graph-theoretical approaches may be helpful. Also, in cases when this level of detail is simply too high, and unnecessary for answering particular questions, graph-theoretical methods can be helpful [8].

Stoichiometric matrices do not capture all the interactions that take place in a network. Regulatory influences where an effector inhibits or activates a reaction without being consumed are not incorporated in the stoichiometric matrix. The matrix that denotes the occurrence of influences of substrates, products, and effectors on rates of reactions is called the *elasticity matrix* [5]. This matrix, often denoted by $\mathbf{\epsilon}$, has the identity of reactions as rows and, as columns, the identity of the variable intermediates in the network. In metabolic control analysis [5], it is defined as $\partial \ln v / \partial \ln s$ where v denotes the rate vector and s the vector of all the concentrations of the variable intermediates. An i th entry in the rate vector contains the rate equation of the i th reaction in the network, e.g., a Michaelis–Menten equation. $\{\mathbf{\epsilon}\}_{ij}$ then quantifies the sensitivity of the i th reaction with respect to the concentration of the j th intermediate when the network prevails in a particular state. The qualitative elasticity matrix $\tilde{\epsilon}$ has -1 , 0 , or 1 as its entries whenever an interaction is inhibitory, non-existing or activatory, respectively [15]. Analyses of the qualitative elasticity matrix may be referred to as *regulatory topological analyses*.

Another matrix that describes the interaction structure of a molecular interaction network is the *Jacobian matrix* $\mathbf{M}$ [5]. It may be defined as the matrix product of $\mathbf{N}\mathbf{\epsilon}$ (strictly, this definition is only correct for systems without moiety conservation). It also represents a description of the topology of the network as a graph. The rows and the columns of this matrix correspond to the identities of the variable intermediates in the network. A non-zero i,j th entry indicates whether there is a direct interaction from intermediate j to i in the network. A direct interaction from intermediate j to i occurs if intermediate j influences any of the production or consumption rates of intermediate i . Formally, $\{\mathbf{M}\}_{ij}$ equals $\partial(ds_i/dt)/\partial s_j$ where ds_i/dt denotes the rate of change in the concentration of intermediate i at time t . Also, this equation can be considered qualitatively by using -1 , 0 , and 1 to denote inhibitory, absent, or activatory interactions. The analysis of this matrix plays a dominant role in sensitivity and stability analysis of kinetic models of molecular reaction networks [16]. The qualitative analysis of the Jacobian matrix is also a kind of *regulatory topological analysis*.

So far all the models discussed concern solely structural aspects of the network. The kinetic properties of the enzymes catalyzing the reactions in the network are not incorporated. Kinetic models, however, do incorporate that kind of information. Such models allow for the calculation of the rate of a particular reaction and, subsequently, the changes in the concentrations of the variable intermediates as they are given by the difference between its net production and consumption rates.

Enzyme Kinetics

Given a stoichiometric description of the network, a *kinetic model* can be constructed by supplying the rate equations for all the reactions considered in the network. A rate equation is a formula expressing the rate of an enzyme in terms of its kinetic parameters and the

concentrations of its substrates, products, and effectors. A rate equation for the k th enzyme-catalyzed reaction, involving n substrates x_i and m products y_j :

has a general structure [17, 18]:

$$v_k = V_{\text{MAX},k}^+ \cdot \prod_{i=1}^n x_i \cdot \left(1 - \frac{\prod_{j=1}^m y_j}{\prod_{i=1}^n x_i \cdot K_{\text{EQ},k}} \right) \cdot f(\mathbf{s}, \mathbf{p}_k) \quad (1)$$

Here $V_{\text{MAX},k}^+$ represents the maximal rate of the enzyme in the direction of the synthesis of the products. This constant can be expressed as $k_{\text{cat},k} \cdot e_k$ with $k_{\text{cat},k}$ and e_k denoting the catalytic rate constant and enzyme concentration, respectively. $K_{\text{EQ},k}$ denotes the equilibrium constant of the enzyme, which can be expressed in terms of the kinetic properties of the enzyme using the Haldane constant [19]. The vector $\mathbf{s}$ contains all the concentrations of the variable intermediates in the network. The vector $\mathbf{p}_k$ harbours all the kinetic parameters of the enzyme. The general structure of this equation can be exploited to obtain information about the regulation of rates in metabolism using hierarchical regulation analysis [20–23]. The function f has a structure that depends on the exact mechanism by which the enzyme converts the substrates into products. Rate equations as expressed in Eq. 1 can be derived using quasi-equilibrium or quasi-steady state models for the enzyme mechanism in terms of elementary reactions [24, 25]. Cleland has worked out many enzyme mechanisms using the so-called King–Altman approach to deriving quasi-steady-state enzyme kinetics [26–28]. Cooperative enzymes, displaying sigmoidal or more complex dependences of the rate as a function of the concentration of substrates, products, and effectors, also follow the structure of Eq. 1 but have more complicated f functions [18, 29–31]. An appealing approximation of cooperative enzyme mechanisms is the reversible Hill equation [18]. Membrane transport by way of active mechanisms and facilitated diffusion can also be modeled with rate equations of the type of Eq. 1. Reactions that involve substrate channeling lead to more complicated equations, and the rate may depend on the product of concentrations of the enzymes directly exchanging intermediates, without equilibrating with their bulk concentrations [32]. Non-enzyme catalyzed reactions can be described by mass-action kinetics. The general description of the rate of such a reaction can be obtained from Eq. 1 by removing e_k and $f(\mathbf{s}, \mathbf{p})$. Note that if the reaction is $2A \leftrightarrow B$, powers appear in the numerator of Eq. 1.

Kinetic Modelling, Coarse Graining, and Choice of Simulation Method

A *kinetic model* is characterized by the network, its stoichiometric matrix, rate vector (containing all the parameterized rate equations), initial conditions, and specification of the environment (identification of fixed concentrations, inflows and outflows). The set of initial conditions is given by the set of concentrations of intermediates at the initial time point of the calculation of the dynamics of the network. The complete set of concentrations is referred to as the *state* of the network. Depending on the physicochemical conditions under which these reactions take place in the organism, the dynamics of the network can then be calculated using particular *simulation methods*. By *dynamics*, we mean the changes of the concentration of the variable intermediates involved in the network as function of time.

Here we shall discuss stochastic and continuous simulation methods for simulating spatiotemporal phenomena.

A functioning network operates in one or more compartments composing a cell. This could be the cytoplasm, an organelle, or a membrane. The molecular components of the network occur in certain abundances, i.e., copy numbers, and those copy numbers change because of processes (transport and reactions). Before a particular reaction can take place, the reactants have to come in close enough proximity to allow for the reaction to occur. Often such transport relies on diffusion. When the reactants collide, the reaction can then occur with a particular probability; depending, for instance, on the orientation of the molecules when they collide [33]. This leads to the net rate of a reaction being limited by the rates of diffusion, by the reaction rate, or by both. In such a single-molecule perspective, the individual motions of molecules are driven by thermal noise (Brownian motion) and the processes between them are dependent on intramolecular ‘noise’ making these processes intrinsically probabilistic [33, 34].

Often it is not the individual molecules that matter but it is the copy numbers of a particular species of molecules we can measure and want to compute. Similarly, the number of reactions occurring of a given type is often of interest rather than individual reactions taking place between particular reactants. A description in those terms would still be probabilistic. It would describe the dynamics of the probability of the network to be in a particular state by calculating the rate of change of this probability due to the occurrence of processes. Such a description is offered by the so-called *master equation* [33, 35–37]. The master equation may incorporate diffusion [38, 39] or it may describe the dynamics of well-stirred systems [35, 40]. The latter description is adopted most often at the present time in biosimulation.

Master equation models are more ‘coarse grained’ (less detailed) than microscopic methods [37]. Microscopic methods follow the fate of individual molecules in the network over time. Master equation models have been termed *mesoscopic*. As far as the authors know, microscopic methods are not extensively used for the biosimulation of networks at the present time.

The most popular simulation method is the use of partial and ordinary differential equations models. Those approaches are *macroscopic* and calculate the change in the state of the network as function of time by treating the state as a vector of non-random continuous variables. Given certain conditions, i.e., when taking particular limits, microscopic methods become mesoscopic methods and mesoscopic methods become macroscopic methods [37].

The thermodynamic limit is involved in approximating a mesoscopic model by a macroscopic model, i.e., going from a master equation model to a differential equation model. We will consider well-stirred systems in this case. The thermodynamic limit is defined as increasing the size of the system, often considered the volume, to infinity, while keeping all the copy numbers in the system divided by the size, often considered the concentration, constant, i.e., $V \rightarrow \infty$ and for all i , $n_i/V \rightarrow \text{constant}$ with V as the volume of the system and n_i as the copy number of molecule i . In this limit, which in practise amounts to having copy numbers higher than ~ 100 for well-behaving systems, e.g., systems removed from bifurcation points, the average obtained from the master equation models corresponds to the solution of the differential equation model and the size of the fluctuations in the master equation model are described up to first order by macroscopic information alone. This approximation has been termed the small noise approximation, linear noise approximation, or omega expansion, and was developed by Van Kampen [37, 41]. Using this method, it appears that fluctuations in metabolic networks can be satisfactorily approxi-

mated. We will not go into more detail here, but refer the reader to the above cited works [37, 41]. A description of the effects of fluctuations that is common in physics, but much less in biology, involves the use of the Langevin equation. For a discussion of its applicability to biological problems, we refer to the work by Gillespie [35].

Master equation models have classically been derived for networks described in terms of mass-action kinetics. In the case of enzyme-catalyzed reactions, this would mean that the elementary reactions taking place in the enzyme's mechanism would have to be modelled explicitly rather than that the enzyme is modelled as a whole by using quasi-equilibrium or quasi-steady state enzyme kinetics. During the last few years, a number of studies have investigated the possibility of enzyme kinetics in master equation models [42, 43].

Kinetic Models Simulated with Ordinary Differential Equations

Here we assume that the thermodynamic limit is valid and that diffusion is sufficiently fast to dissipate spatial gradients in concentrations such that their effects on the reaction rates can be neglected. Given the stoichiometric matrix and the rate vector containing all the parameterized rate equations of the reactions considered in the network, the rate of change of all the variable intermediates in the network is described by:

$$\frac{d}{dt} \mathbf{s}(t, \mathbf{p}, \mathbf{s}_0) = \mathbf{N} \mathbf{v}(\mathbf{s}(t, \mathbf{p}, \mathbf{s}_0), \mathbf{p}) \Rightarrow \frac{d}{dt} \mathbf{s} = \mathbf{N} \mathbf{v} \quad (2)$$

with t denoting time [5]. The dynamics of the system can now be calculated by integration of Eq. 2, given a suitable set of initial conditions:

$$\mathbf{s}_0 = \mathbf{s}(0, \mathbf{p}). \quad (3)$$

The integration procedure then simply involves the equation:

$$\mathbf{s}(t, \mathbf{p}, \mathbf{s}_0) = \mathbf{s}_0 + \int_0^t \frac{d}{d\tau} \mathbf{s}(\tau, \mathbf{p}, \mathbf{s}_0) \cdot d\tau. \quad (4)$$

This set differential equations and initial conditions is an example of a kinetic model described in terms of ordinary differential equations. This constitutes a very coarse grained description of the dynamics of networks. Most of the theory for the analysis of networks has been derived for such formulations.

Silicon Cell Models

Silicon cell models are kinetic models simulated with stochastic or continuous methods that aim at describing the cellular phenomena as precisely as possible given the current available knowledge about the characterization of the network, i.e., its topology, kinetic description of processes, total concentrations of moieties, and communication with the environment. This does not mean that silicon cell models are the final model of the system; they are simply the best description we can give at the present time. Silicon cell models are descriptive and may therefore be highly complicated. Often model reduction strategies may be helpful to offer explanations of particular phenomena. A number of silicon cell models exist at the present time and their number is rising (for a collection of models that can be

explored online, the reader is referred to JWS online at www.jjj.bio.vu.nl [44]. The first silicon cell models were built in the 1960–1970s [45–47].

Some of the advantages of silicon cell models are: (a) they offer opportunities to discover new regulatory mechanisms [48], (b) they allow for testing alternative experimental designs not readily constructible in the laboratory [49], (c) they offer predictions of interest to bioengineering and drug design [50, 51], (d) they constitute a test of the consistency of our understanding as they allow for the calculation of physiological behaviour given our knowledge of the underlying biochemistry [52–55], (e) they offer an opportunity to integrate data obtained in large-scale, high-throughput “Omics” experiments, and (f) they allow for a modular construction of a silicon cell model for an entire cell [56].

Among the disadvantages of silicon cell models, we may mention: (a) their large size in terms of the number of variables, reactions, and parameters, which makes them hard to analyze and verify, (b) the significant number of kinetic experiments necessary to parameterize all the rate equations, and (c) the fact that all biochemical phenomena have not yet been characterized in sufficient detail, particularly phenomena related to transcriptional and translational regulation.

The alternative to silicon cell models is phenomenological kinetic models ranging from core models to quasi-detailed models. Core models are useful for a qualitative illustration or explanation of a particular phenomenon. They were used frequently in the 1970s, e.g., [57, 58]. This approach is very appealing because of its use of simple models, i.e., a small number of variables and reactions. It has been used quite often in the past. The disadvantage is that mapping of the simplified network, described with the core model, onto the *in vivo* network is not unambiguous. The kinetic parameters and rate equations are often phenomenological, which makes it hard to test predictions even qualitatively.

One approach to parameterizing rate equations is by measuring the kinetic parameters of enzymes in isolation, either in cell-free extract or by using purified enzymes. Experimental design methods have been developed that allow for the identification of the enzyme mechanism and its kinetic characterization [26–28]. Many of these approaches can also be found in textbooks on enzyme kinetics [24, 25]. These methods have been applied to many (mostly metabolic) enzymes in the seventies and eighties. This body of literature offers a rich source of kinetic data for the construction of silicon cells and quasi-detailed models.

Another approach to parameterize rate equations is by *parameter estimation* methods [59–61]. With such methods, parameters for which the value is unknown are varied in a kinetic model according to particular algorithms and, subsequently, the kinetic model is sought for that best describes a set of experimental data. Often multiple parameter sets describe the same data set and additional experiments are necessary to distinguish between alternative models. Especially at the present time, with the rise of more quantitative large-scale high throughput experiments of system data such as metabolites, mRNA, protein and fluxes, we anticipate that parameter estimation will become more popular. However, studies on enzymes in isolation seem to offer many advantages. Even when one acknowledges the possible discrepancies between *in vitro* and *in vivo* kinetic parameter values, *in vitro* kinetic parameters will be a promising initial guess for parameter estimation studies.

An Example of a Silicon Cell Model

Ammonium is the preferred nitrogen source of *Escherichia coli*; implying that the highest growth rate is obtained with ammonium as nitrogen source and all other nutrients in excess.

This bacterium has two mechanisms at its disposal to assimilate ammonium [62]. One involving glutamine synthetase (GS) and glutamate synthase (GOGAT) and the other involves glutamate dehydrogenase (GDH). These two mechanisms are under the control of a very complicated molecular network involving: a covalent modification cascade [63–67], inducible expression of high and low affinity assimilation pathways [62, 68], an expressible facilitated-transport system [68], feedback inhibition [69, 70], two-component signal transduction [63, 64], and oligomeric enzymes [62]. For a recent review, focussing predominantly on the molecular properties of this network, the reader is referred to Ninfa *et al.* [71].

Recently, we developed a detailed kinetic model of this regulatory network considering all metabolic processes [53]. This model was based on literature data on the kinetic properties of the molecular components of the system. It did not incorporate the two-component signalling system and regulation by gene expression. This model represents a silicon cell model because it contains our complete molecular understanding, to the extent that this is available in the literature at the present time.

The model incorporates the covalent-modification cascade and describes the regulation of the partitioning of the ammonium assimilation uptake rate over GS and GDH (Figure 1). GS has a higher affinity for ammonium than GDH, making it the preferred uptake system during conditions of ammonium limitation [72]. Usage of GS comes at the cost of the additional hydrolysis of one molecule of ATP per molecule of ammonium. Most likely because of this [73–75], *E. coli* expresses GDH at higher concentrations of ammonium. Under such conditions, in the absence of the covalent modification cascade, GS would be operative at its maximal rate—being saturated with ammonium—while GDH is moderately to maximally active [76]. To prevent the high activity of GS, and concomitant high ATP expenditure, GS is under strict control by at least two mechanisms. The first mechanism is the reversible inactivation of GS by covalent adenylation of any of its 12 subunits by adenylyltransferase (ATase) [65, 76]. ATase is a bifunctional enzyme that can adenylylate and deadenylylate GS depending on the levels of various effectors [53, 63, 64]. The adenylylation state of GS increases with the ammonium concentration [53] and causes the maximal rate of GS to decrease [69, 70]. The second mechanism involves a reduced expression of GS at high concentrations of ammonium; it only has minor cooperative effects between the substrates, products, and effectors of GS [69, 70]. Under conditions of ammonium deprivation, GS is expressed at high levels and, in addition, an ammonium transporter is expressed to scavenge ammonium [68, 77]. This transporter is under the control of the regulatory protein GlnK [78]. GlnK is a homologue of PII [79]. PII is a regulatory protein that is involved in regulating the activity of ATase and can be triply uridylylated depending on the levels of its effectors glutamine and 2-oxoglutarate. In addition, PII acts as the signal for the two-component signalling network NRI-NRII when it is complexed with one molecule of 2-oxoglutarate and is in its un-uridylylated form. This signalling system senses the internal nitrogen and carbon status and regulates the activity of the operons involved in the Ntr regulon [80]; including the operon encoding GS, NRI and NRII.

All in all, this system has a molecular complexity that defies understanding by the human mind if this is to be achieved by staring at the molecular interaction network scheme alone (displayed in Figure 1)—this system incorporates multiple parallel regulatory routes that may act antagonistically or synergistically depending on conditions [53]. Considering that this system has already been studied molecularly for over 30 years [81], it seems an ideal system for the construction of a silicon cell model. (For the same reasons that detailed models have been developed for glycolysis in erythrocytes [47], *E. coli* [82], yeast [55], and trypanosomes [52]).

The following calculations with the model serve to illustrate the power of the simultaneous use of theory when analyzing kinetic models. Without theory, analysis of kinetic models is nothing more than simulation, which may help formulate hypothesis and highlight unanticipated phenomena but is limited when one wants to explain and come to understand model behaviour.

When analyzing our model of ammonium assimilation we were struck by the near constancy of net steady-state ammonium assimilation flux J_N , the sum of the flux through GS and GDH, as a function of the ammonium concentration. It turned out that any increase in the concentration of ammonium lead to an increase in the GDH flux and a resultant rise in the glutamine level, which activated the adenylation rate of ATase. This, in turn, caused the maximal rate of GS to decrease. In this manner, the network counteracts, almost perfectly, the initial increase in the rate of GS caused by an increase in the concentration of its substrate, ammonium. This phenomenon resembles integral control [83, 84], and this will be studied more carefully elsewhere. One aspect we did not analyze in our previous studies was the control exerted by the enzymes in

Figure 1 The network structure of the regulatory processes involved in ammonium assimilation by *E. coli* at a metabolic time scale. Reactions, accompanied by enzyme names, are denoted by *solid arrows*. Dashed arrows display regulatory interactions (simplified; cf. Bruggeman and Boogerd [53]). For clarity, the demand pathways for glutamate, GLNDEM and AZGLNDEM, are not displayed. Variable intermediates are in *italic font* whereas boundary metabolites are denoted in *normal font*.

the network on the ratio of the fluxes of GS and GDH, i.e., J_{GS}/J_{GDH} . This is what we set out to do in what follows.

The ratio of J_{GS} over J_{GDH} is of interest because the sum of J_{GS} and J_{GDH} , which equals J_N , proved robust with respect to changes in the ammonium concentration. Apparently, this ratio is under strict control. We also chose to consider this ratio to highlight the power of the simultaneous use of theory when analyzing control properties of biochemical reaction networks.

Table I displays the values of the control coefficients for all enzymes in the system on this flux ratio. Here we have defined a control coefficient for a process j , e.g., an enzyme-catalyzed reaction, on the steady-state systemic property f as [85–87],

$$C_j^f = \frac{d \ln f}{d \ln \lambda_j}.$$

The constant λ_j acts as a multiplier with which we multiply the rate of enzyme j while the system prevails in steady state and then allow the system to evolve to a new steady state. Thus, we have to assume stability of the reference steady state. We observe that GDH has the control over this ratio, causing a near proportional decrease in J_{GS}/J_{GDH} following an increase in activity. GOGAT has a moderate positive control followed by the demand pathways for glutamine and glutamate.

When studying Table I more closely, two facts become apparent. Firstly, the sum of all the control coefficients equals zero exactly. Secondly, four pairs of control coefficients sum to zero exactly. These facts deserve explanation.

We recognize that the following relationship holds (because $\ln(x) - \ln(y) = \ln(x/y)$),

$$C_i^{J_{GS}/J_{GDH}} = C_i^{J_{GS}} - C_i^{J_{GDH}}.$$

The summation theorem for flux control coefficients dictates that the sum of the control coefficients of all processes on the same flux J_j equals 1 (r equals the number of processes in the system) [85, 86],

$$\sum_{i=1}^r C_i^{J_j} = 1.$$

Table I The control coefficients for all enzymes in the network on the ratio J_{GS}/J_{GDH}

Enzyme	$C_{enzyme}^{J_{GS}/J_{GDH}}$
GS	0.02
GOGAT	0.38
GDH	−1.1
GLNDEM	0.169
GLUDEM	−0.168
AZGLNDEM	0.18
AZGLUDEM	0.50
ATPase	0.02
UT1	0.04
UR1	−0.04
UT2	0.05
UR2	−0.05
UT3	0.07
UR3	−0.07
AD	−0.08
DEAD	0.08
Sum	0

The model as reported in Bruggeman and Boogerd [53] was used with ammonium and 2-oxoglutarate concentrations equal to 0.5 and 1.0 mM, respectively.

Therefore, we conclude that

$$\sum_{i=1}^r C_i^{J_k/J_l} = \sum_{i=1}^r C_i^{J_k} - \sum_{i=1}^r C_i^{J_l} = 0.$$

This explains the first observation. Note that, without knowledge of the summation theorems, this relationship might have gone unnoticed. It represents a constraint on the value of the sum of the control coefficients. It indicates that the flux ratio does not change when rescaling time in the model [87]. The second observation involved the identification of four pairs of control coefficients that each sum to zero exactly, i.e., $C_{UT1}^{J_{GS}/J_{GDH}} = -C_{UR1}^{J_{GS}/J_{GDH}}$, $C_{UT2}^{J_{GS}/J_{GDH}} = -C_{UR2}^{J_{GS}/J_{GDH}}$, $C_{UT3}^{J_{GS}/J_{GDH}} = -C_{UR3}^{J_{GS}/J_{GDH}}$, and $C_{AD}^{J_{GS}/J_{GDH}} = -C_{DEAD}^{J_{GS}/J_{GDH}}$. Close investigation of the system reveals that, in steady state, the individual enzymes that make up these pairs form together an independent flux mode and that no other fluxes in the system are a part of that flux mode. This property of biochemical networks with a “cascade”-type or “level”-type of organization was first identified by Kahn and Westerhoff [88]. Kahn and Westerhoff showed that the sum of the control coefficients of those pairs of the fluxes in the flux mode equals 1 and, on fluxes not part of the flux, equals 0. Since, GS and GDH carry different fluxes in steady state than UT1, UR1, UT2, UR2, UT3, UR3, AD and DEAD, the abovementioned relationship between the control coefficients was found. Again, this invariant property of the network would have gone unnoticed without the existence of theory. Interestingly, these two observations are independent of the steady state of the system; they would appear again if changes in parameters were applied. The existence of such relationships indicates that the number of independent control coefficients may be considerably smaller than the number of fluxes in the network under consideration [5, 88].

Theory Development

Another aspect of biosimulation is the development of theory that facilitates: (a) the integration of model with experiment, (b) the construction, verification, and analysis of kinetic models, and (c) discovery of general principles governing cellular phenomena. A large body of theory has been developed over the years that, in some way or another, is applicable to studies of molecular interaction networks. Here we shall only discuss a small number of examples relating to the analysis of kinetic models. For the analysis of stoichiometric models the reader is referred to Price *et al.* [7]. *Metabolic control analysis* (MCA), and its extensions *modular* and *hierarchical control analysis*, allow for the quantification of the importance of particular reactions for bringing about changes in systemic properties in molecular interaction networks. Reactions with high importance are then said to be controlling and the amount of control is quantified in terms of a control coefficient. For example, a flux control coefficient $C_{v_i}^{J_j}$ is defined as the fractional change in the control of a flux J_j upon a fractional change in the activity v_i of reaction i . Originally, MCA was developed for the analysis for metabolic systems [85, 86, 89]. Later, MCA was generalized to cover networks of any complexity [5], networks containing particular modular structures [80, 90] and networks composed of levels, e.g., gene expression and signalling subsystems [88, 91–93]. The application of control analysis to time-dependent systemic properties of networks is a continuing endeavour [94–99]. Control analysis also led to the discovery of invariant relationships (laws) between control and elasticity coefficients expressed in terms of summation and connectivity theorems. Whereas the former can be extended to cover time-dependent phenomena, the latter appears to be

exclusively valid for steady-state systems [87, 96, 97]. A related approach, but different in its details, for the analysis of molecular interaction networks is *biochemical systems theory* [100, 101].

Parameter sensitivity analysis is a method that allows for the calculation of the importance of particular parameters for systemic phenomena. It is related to control analysis but differs in spirit as it does not aim at understanding how the design and kinetic properties of networks determine their control and regulatory properties. It also lacks the summation and connectivity theorems of MCA.

Bifurcation analysis is a method used to discover qualitative changes in the (dynamic) system behaviour of molecular interaction networks as function of parameter values [102]. Using this method, the location in parameter space of transitions between steady states (fixed points), to oscillatory dynamics, and chaotic dynamics, can be discovered and analyzed. For an example applied to glycolysis see [103, 104] and for applications to bursting cells, see, for example, [105] and [106].

Hierarchical regulation analysis is a method that allows one to quantify the contribution of regulatory processes to adaptive changes of molecular interaction networks [20–23]. It offers the possibility of integrating experimental data obtained with large-scale high throughput studies involving measurement of levels of fluxes, metabolites, proteins, and mRNA. Although it is related to MCA, hierarchical regulation analysis has several advantages: (a) it is applicable to study the responses to large changes in parameter values, (b) it can be extended to time-dependent phenomena, (c) it is readily applicable to the analysis of experimental data, and (d) it offers an integrative picture of data sets deriving from the “Omics”.

Conclusion

Biosimulation has established itself a firm position in the biological sciences and, in particular, in systems biology [107–109]. This is not so surprising, given the opportunities it offers for rational approaches to bioengineering, medicine (drug design), the design of scientific experiments, and integration of experimental data. Considering the present development of systems biology, biosimulation appears here to stay and we anticipate it to become a mainstream method in (cell) biology. We hope to have given the reader in this review an impression of the versatility of the approaches to biosimulation currently at the disposal of the biologist.

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References

1. Brazhe, N.A., Brazhe, A.R., et al.: Unraveling cell processes: interference imaging weaved with data analysis. In press (2006)
2. Diderichsen, P., Göpel, S.O.: Modeling the electrical activity of pancreatic α -cells based on experimental data from intact mouse islets. In press (2006)
3. Mogilevskaya, E., Demin, O., et al.: Kinetic model of mitochondrial Krebs cycle: unravelling the mechanism of salicylate hepatotoxic effects. In press (2006)
4. Rapin, N., Kesmir, C., et al.: Integrating bioinformatics and systems biology for immune system modeling. In press (2006)
5. Reder, C.: Metabolic control theory: a structural approach. *J. Theor. Biol.* **135**(2), 175–201 (1988)
6. Papin, J.A., Stelling, J., et al.: Comparison of network-based pathway analysis methods. *Trends Biotechnol.* **22**(8), 400–405 (2004)

7. Price, N.D., Reed, J.L., et al.: Genome-scale models of microbial cells: evaluating the consequences of constraints. *Nat. Rev. Microbiol.* **2**(11), 886–897 (2004)
8. Jeong, H., Tombor, B., et al.: The large-scale organization of metabolic networks. *Nature* **407**(6804), 651–654 (2000)
9. Wagner, A., Fell, D.A.: The small world inside large metabolic networks. *Proc. R. Soc. Lond. B Biol. Sci.* **268**(1478), 1803–1810 (2001)
10. Albert, R., Barabasi, A.L.: Statistical mechanics of complex networks. *Rev. Mod. Phys.* **74**, 47–97 (2002)
11. Milo, R., Shen-Orr, S., et al.: Network motifs: simple building blocks of complex networks. *Science* **298**(5594), 824–827 (2002)
12. Newman, M.E.J.: The structure and function of complex networks. *SIAM Rev.* **45**, 167–256 (2003)
13. Hornberg, J., Binder, B., Bruggeman, F.J., Schoeberl, B., Heinrich, R., Westerhoff, H.V.: Who is in control of MAPK signaling? Manuscript in preparation (2004)
14. Kholodenko, B.N., Demin, O.V., et al.: Quantification of short term signaling by the epidermal growth factor receptor. *J. Biol. Chem.* **274**(42), 30169–30181 (1999)
15. Schuster, S., Schuster, R.: Decomposition of biochemical reaction systems according to flux control insusceptibility. *J. Chim. Phys.* **89**(9), 1887–1910 (1992)
16. Stucki, J.W.: Stability analysis of biochemical systems—practical guide. *Prog. Biophys. Mol. Biol.* **33**(2), 99–187 (1978)
17. Heinrich, R., Schuster, S.: *The Regulation of Cellular Systems*. Chapman & Hall, New York (1996)
18. Hofmeyr, J.H., Cornish-Bowden, A.: The reversible Hill equation: how to incorporate cooperative enzymes into metabolic models. *Comput. Appl. Biosci.* **13**(4), 377–385 (1997)
19. Cleland, W.W.: An analysis of Haldane relationships. *Methods Enzymol.* **87**, 366–369 (1982)
20. Even, S., Lindley, N.D., et al.: Transcriptional, translational and metabolic regulation of glycolysis in *Lactococcus lactis* subsp. *cremoris* MG 1363 grown in continuous acidic cultures. *Microbiology* **149**(Pt 7), 1935–1944 (2003)
21. ter Kuile, B.H., Westerhoff, H.V.: Transcriptome meets metabolome: hierarchical and metabolic regulation of the glycolytic pathway. *FEBS Lett.* **500**(3), 169–171 (2001)
22. Rossell, S., van der Weijden, C.C., et al.: Hierarchical and metabolic regulation of glucose influx in starved *Saccharomyces cerevisiae*. *FEMS Yeast Research* **5**(6–7), 611–619 (2005)
23. Rossell, S., van der Weijden, C.C., et al.: Unraveling the complexity of flux regulation: a new method demonstrated for nutrient starvation in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **103**(7), 2166–2171 (2006)
24. Cornish-Bowden, A.: *Fundamentals of Enzyme Kinetics*. Portland, London (1995)
25. Segel, I.H.: *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. Wiley (1993)
26. Cleland, W.W.: The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. *Biochim. Biophys. Acta* **67**, 104–137 (1963)
27. Cleland, W.W.: The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. *Biochim. Biophys. Acta* **67**, 173–187 (1963)
28. Cleland, W.W.: The kinetics of enzyme-catalyzed reactions with two or more substrates or products. III. Prediction of initial velocity and inhibition patterns by inspection. *Biochim. Biophys. Acta* **67**, 188–196 (1963)
29. Koshland, D.E., Jr., Nemethy, G., et al.: Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* **5**(1), 365–385 (1966)
30. Monod, J., Wyman, J., et al.: On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* **12**, 88–118 (1965)
31. Popova, S.V., Sel'kov, E.E.: Generalization of the model by Monod, Wyman and Changeux for the case of a reversible monosubstrate reaction SR,TP. *FEBS Lett.* **53**(3), 269–273 (1975)
32. van Dam, K., van der Vlag, J., et al.: The sum of the control coefficients of all enzymes on the flux through a group-transfer pathway can be as high as two. *Eur. J. Biochem.* **212**(3), 791–799 (1993)
33. Keizer, J.: *Statistical Thermodynamics of Nonequilibrium Processes*. Springer, Berlin (1987)
34. Rao, C.V., Wolf, D.M., et al.: Control, exploitation and tolerance of intracellular noise. *Nature* **420**(6912), 231–237 (2002)
35. Gillespie, D.T.: A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. *J. Comput. Phys.* **22**, 403–434 (1976)
36. McQuarrie, D.A.: Stochastic approach to chemical kinetics. *J. Appl. Probab.* **4**, 413–478 (1967)
37. Van Kampen, N.G.: *Stochastic Processes in Chemistry and Physics*. North-Holland, Amsterdam (1992)
38. Baras, F., Mansour, M.M.: Reaction-diffusion master equation: a comparison with microscopic simulations. *Phys. Rev. E Stat. Phys. Plasmas Fluids Relat. Interdiscip. Topics* **54**(6), 6139–6148 (1996)
39. Hattne, J., Fange, D., et al.: Stochastic reaction-diffusion simulation with MesoRD. *Bioinformatics* **21**(12), 2923–2924 (2005)

40. Arkin, A., Ross, J., et al.: Stochastic kinetic analysis of developmental pathway bifurcation in phage lambda-infected *Escherichia coli* cells. *Genetics* **149**(4), 1633–1648 (1998)
41. Elf, J., Ehrenberg, M.: Fast evaluation of fluctuations in biochemical networks: with the linear noise approximation. *Genome Res.* **13**(11), 2475–2484 (2003)
42. Cao, Y., Gillespie, D.T., et al.: Accelerated stochastic simulation of the stiff enzyme-substrate reaction. *J. Chem. Phys.* **123**, 144917 (2005)
43. Rao, C.V., Arkin, A.P.: Stochastic chemical kinetics and the quasi-steady-state assumption: application to the Gillespie algorithm. *J. Chem. Phys.* **118**(11), 4999–5010 (2003)
44. Olivier, B.G., Snoep, J.L.: Web-based kinetic modelling using JWS online. *Bioinformatics* **20**(13), 2143–2144 (2004)
45. Chance, B., Garfinkel, D., et al.: Metabolic control mechanisms. 5. A solution for the equations representing interaction between glycolysis and respiration in ascites tumor cells. *J. Biol. Chem.* **235**, 2426–2439 (1960)
46. Garfinkel, D., Hess, B.: Metabolic control mechanisms. vii. A detailed computer model of the glycolytic pathway in ascites cells. *J. Biol. Chem.* **239**, 971–983 (1964)
47. Rapoport, T.A., Heinrich, R., et al.: Linear steady-state treatment of enzymatic chains—mathematical-model of glycolysis of human erythrocytes. *Eur. J. Biochem.* **42**(1), 107–120 (1974)
48. Teusink, B., Walsh, M.C., et al.: The danger of metabolic pathways with turbo design. *Trends Biochem. Sci.* **23**(5), 162–169 (1998)
49. Bakker, B.M., Mensonides, F.I., et al.: Compartmentation protects trypanosomes from the dangerous design of glycolysis. *Proc. Natl. Acad. Sci. USA* **97**(5), 2087–2092 (2000)
50. Hoefnagel, M.H., Starrenburg, M.J., et al.: Metabolic engineering of lactic acid bacteria, the combined approach: kinetic modelling, metabolic control and experimental analysis. *Microbiology* **148**(Pt 4), 1003–1013 (2002)
51. Michels, P.A.M., Bakker, B.M., Opperdoes, F.R., Westerhoff, H.V.: On the mathematical modelling of metabolic pathways and its use in the identification of the most suitable drug target. In: Vial, H., Fairlamb, A., Ridley, R. (eds.) *Tropical Disease Guidelines and Issues: Discoveries and Drug Development*. WHO, Geneva (in press)
52. Bakker, B.M., Michels, P.A.M., et al.: Glycolysis in bloodstream form *Trypanosoma brucei* can be understood in terms of the kinetics of the glycolytic enzymes. *J. Biol. Chem.* **272**(6), 3207–3215 (1997)
53. Bruggeman, F.J., Boogerd, F.C., et al.: The multifarious short-term regulation of ammonium assimilation of *Escherichia coli*: dissection using an in silico replica. *FEBS J.* **272**(8), 1965–1985 (2005)
54. Rohwer, J.M., Meadow, N.D., et al.: Understanding glucose transport by the bacterial phosphoenolpyruvate:glucose phosphotransferase system on the basis of kinetic measurements in vitro. *J. Biol. Chem.* **275**(45), 34909–34921 (2000)
55. Teusink, B., Passarge, J., et al.: Can yeast glycolysis be understood in terms of in vitro kinetics of the constituent enzymes? Testing biochemistry. *Eur. J. Biochem.* **267**(17), 5313–5329 (2000)
56. Snoep, J.L., Bruggeman, F., et al.: Towards building the silicon cell: a modular approach. *Biosystems* **83**(2–3), 207–216 (2006)
57. Goldbeter, A., Lefever, R.: Dissipative structures for an allosteric model. Application to glycolytic oscillations. *Biophys. J.* **12**(10), 1302–1315 (1972)
58. Selkov, E.: Stabilization of energy charge, generation of oscillations and multiple steady states in energy metabolism as a result of purely stoichiometric regulation. *Eur. J. Biochem.* **59**(1), 151–157 (1975)
59. Kremling, A., Fischer, S., et al.: A benchmark for methods in reverse engineering and model discrimination: problem formulation and solutions. *Genome Res.* **14**(9), 1773–1785 (2004)
60. Mendes, P., Kell, D.: Non-linear optimization of biochemical pathways: applications to metabolic engineering and parameter estimation. *Bioinformatics* **14**(10), 869–883 (1998)
61. Moles, C.G., Mendes, P., et al.: Parameter estimation in biochemical pathways: a comparison of global optimization methods. *Genome Res.* **13**(11), 2467–2474 (2003)
62. Rhee, S.G., Chock, P.B., et al.: Regulation of *Escherichia coli* glutamine synthetase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **62**, 37–92 (1989)
63. Jiang, P., Peliska, J.A., et al.: Reconstitution of the signal-transduction bicyclic cascade responsible for the regulation of Ntr gene transcription in *Escherichia coli*. *Biochemistry* **37**(37), 12795–12801 (1998)
64. Jiang, P., Peliska, J.A., et al.: The regulation of *Escherichia coli* glutamine synthetase revisited: role of 2-ketoglutarate in the regulation of glutamine synthetase adenylylation state. *Biochemistry* **37**(37), 12802–12810 (1998)
65. Kustu, S., Hirschman, J., et al.: Covalent modification of bacterial glutamine synthetase: physiological significance. *Mol. Gen. Genet.* **197**(2), 309–317 (1984)

66. Rhee, S.G., Park, R., et al.: Allosteric regulation of monocyclic interconvertible enzyme cascade systems: use of *Escherichia coli* glutamine synthetase as an experimental model. *Proc. Natl. Acad. Sci. USA* **75**(7), 3138–3142 (1978)
67. van Heeswijk, W.C., Wen, D., et al.: The *Escherichia coli* signal transducers PII (GlnB) and GlnK form heterotrimers in vivo: fine tuning the nitrogen signal cascade. *Proc. Natl. Acad. Sci. USA* **97**(8), 3942–3947 (2000)
68. Javelle, A., Severi, E., et al.: Ammonium sensing in *Escherichia coli*. Role of the ammonium transporter AmtB and AmtB–GlnK complex formation. *J. Biol. Chem.* **279**(10), 8530–8538 (2004)
69. Denton, M.D., Ginsburg, A.: Some characteristics of the binding of substrates of glutamine synthetase from *Escherichia coli*. *Biochemistry* **9**(3), 617–632 (1970)
70. Ginsburg, A., Yeh, J., et al.: Some effects of adenylylation on the biosynthetic properties of the glutamine synthetase from *Escherichia coli*. *Biochemistry* **9**(3), 633–649 (1970)
71. Ninfa, A.J., Jiang, P., et al.: Integration of antagonistic signals in the regulation of nitrogen assimilation in *Escherichia coli*. *Curr. Top. Cell. Regul.* **36**, 31–75 (2000)
72. Rhee, S.G., Chock, P.B., et al.: Glutamine synthetase from *Escherichia coli*. *Methods Enzymol.* **113**, 213–241 (1985)
73. Helling, R.B.: Why does *Escherichia coli* have two primary pathways for synthesis of glutamate? *J. Bacteriol.* **176**(15), 4664–4668 (1994)
74. Helling, R.B.: Pathway choice in glutamate synthesis in *Escherichia coli*. *J. Bacteriol.* **180**(17), 4571–4575 (1998)
75. Helling, R.B.: Speed versus efficiency in microbial growth and the role of parallel pathways. *J. Bacteriol.* **184**(4), 1041–1045 (2002)
76. Kustu, S., Hirschman, J., et al.: Adenylylation of bacterial glutamine synthetase: physiological significance. *Curr. Top. Cell. Regul.* **27**, 201–213 (1985)
77. Coutts, G., Thomas, G., et al.: Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB. *EMBO J.* **21**(4), 536–545 (2002)
78. van Heeswijk, W.C., Hoving, S., et al.: An alternative PII protein in the regulation of glutamine synthetase in *Escherichia coli*. *Mol. Microbiol.* **21**(1), 133–146 (1996)
79. Ninfa, A.J., Atkinson, M.R.: PII signal transduction proteins. *Trends Microbiol.* **8**(4), 172–179 (2000)
80. Zimmer, D.P., Soupene, E., et al.: Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. *Proc. Natl. Acad. Sci. USA* **97**(26), 14674–14679 (2000)
81. Stadtman, E.R.: The story of glutamine synthetase regulation. *J. Biol. Chem.* **276**(48), 44357–44364 (2001)
82. Chassagnole, C., Noisommit-Rizzi, N., et al.: Dynamic modeling of the central carbon metabolism of *Escherichia coli*. *Biotechnol. Bioeng.* **79**(1), 53–73 (2002)
83. Sontag, E.: Adaptation and regulation with signal detection implies internal model. *Syst. Control Lett.* **50**, 119–126 (2003)
84. Yi, T.M., Huang, Y., et al.: Robust perfect adaptation in bacterial chemotaxis through integral feedback control. *Proc. Natl. Acad. Sci. USA* **97**(9), 4649–4653 (2000)
85. Heinrich, R., Rapoport, T.A.: A linear steady-state treatment of enzymatic chains. General properties, control and effector strength. *Eur. J. Biochem.* **42**(1), 89–95 (1974)
86. Kacser, H., Burns, J.A.: The control of flux. *Symp. Soc. Exp. Biol.* **27**, 65–104 (1973)
87. Westerhoff, H.V., Van Dam, K.: *Thermodynamics and Control of Biological Free-Energy Transduction*. Elsevier (Biomedical Division), Amsterdam (1987)
88. Kahn, D., Westerhoff, H.V.: Control theory of regulatory cascades. *J. Theor. Biol.* **153**(2), 255–285 (1991)
89. Fell, D.A.: *Understanding the Control of Metabolism*. Portland, London (1997)
90. Schuster, S., Kahn, D., et al.: Modular analysis of the control of complex metabolic pathways. *Biophys. Chem.* **48**(1), 1–17 (1993)
91. Bruggeman, F.J., Westerhoff, H.V., et al.: Modular response analysis of cellular regulatory networks. *J. Theor. Biol.* **218**(4), 507–520 (2002)
92. Hofmeyr, J.H., Westerhoff, H.V.: Building the cellular puzzle: control in multi-level reaction networks. *J. Theor. Biol.* **208**(3), 261–285 (2001)
93. Westerhoff, H.V., Koster, J.G., van Workum, M., Rudd, K.E.: On the control of gene expression. In: Cornish-Bowden, A., Cardenas, M.L. (eds.) *Control of Metabolic Processes*, pp. 399–413. Plenum, New York (1989)
94. Acerenza, L., Sauro, H.M., et al.: Control analysis of time-dependent metabolic systems. *J. Theor. Biol.* **137**(4), 423–444 (1989)

95. Demin, O.V., Westerhoff, H.V., et al.: Control analysis of stationary forced oscillations. *J. Phys. Chem. B* **103**(48), 10695–10710 (1999)
96. Hornberg, J.J., Bruggeman, F.J., et al.: Principles behind the multifarious control of signal transduction. ERK phosphorylation and kinase/phosphatase control. *FEBS J.* **272**(1), 244–258 (2005)
97. Ingalls, B.P., Sauro, H.M.: Sensitivity analysis of stoichiometric networks: an extension of metabolic control analysis to non-steady state trajectories. *J. Theor. Biol.* **222**(1), 23–36 (2003)
98. Kholodenko, B.N., Demin, O.V., et al.: Control analysis of periodic phenomena in biological systems. *J. Phys. Chem. B* **101**(11), 2070–2081 (1997)
99. Peletier, M.A., Westerhoff, H.V., et al.: Control of spatially heterogeneous and time-varying cellular reaction networks: a new summation law. *J. Theor. Biol.* **225**(4), 477–487 (2003)
100. Savageau, M.A.: *Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology*. Addison-Wesley, New York (1976)
101. Voit, E.O.: *Computational Analysis of Biochemical Systems: A Practical Guide for Biochemists and Molecular Biologists*. Cambridge University Press, Cambridge (2000)
102. Wiggins, S.: *Introduction to Applied Nonlinear Dynamics and Chaos*. Springer, New York (2003)
103. Hynne, R., Dano, S., et al.: Full-scale model of glycolysis in *Saccharomyces cerevisiae*. *Biophys. Chemist.* **94**(1–2), 121–163 (2001)
104. Reijenga, K.A., van Megen, Y.M., et al.: Yeast glycolytic oscillations that are not controlled by a single oscillator: a new definition of oscillator strength. *J. Theor. Biol.* **232**(3), 385–398 (2005)
105. Mosekilde, E., Landing, B., et al.: Bifurcation structure of a model of bursting pancreatic cells. *BioSystems* **63**, 3–6 (2001)
106. Postnov, D.E., Ryazanova, L.S., et al.: Neural synchronization via potassium signaling. *Int. J. Neural Syst.* **16**, 99–109 (2006)
107. Alberghina, L., Westerhoff, H.V. (eds.): *Systems Biology: Definitions and Perspectives (Topics in Current Genetics)*. Springer, Berlin (2005)
108. Kriete, A., Eils, R. (eds.): *Computational Systems Biology*. Elsevier Academic, London, UK (2005)
109. Westerhoff, H.V., Palsson, B.O.: The evolution of molecular biology into systems biology. *Nat. Biotechnol.* **22**(10), 1249–1252 (2004)