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Advantages and disadvantages of aggregating fluxes into synthetic and degradative fluxes when modelling metabolic pathways

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It is now widely accepted that mathematical models are needed to predict the behaviour of complex metabolic networks in the cell, in order to have a rational basis for planning metabolic engineering with biotechnological or therapeutical purposes. The great complexity of metabolic networks makes it crucial to simplify them for analysis, but without violating key principles of stoichiometry or thermodynamics. We show here, however, that models for branched complex systems are sometimes obtained that violate the stoichiometry of fluxes at branch points and as a result give unrealistic metabolite concentrations at the steady state. This problem is especially important when models are constructed with the S-system form of biochemical systems theory. However, the same violation of stoichiometry can occur in metabolic control analysis if control coefficients are assumed to be constant when trying to predict the effects of large changes. We derive the appropriate matrix equations to analyse this type of problem systematically and to assess its extent in any given model.

Keywords: flux aggregation, metabolic control analysis, biochemical systems theory, metabolism, kinetic models.

The metabolic activities of living cells are accomplished by a regulated and highly coupled network of enzyme-catalysed reactions and selective transport systems. The complexity of the networks makes it necessary to construct models to understand and predict their behaviour, but these are always simplifications of reality. Several approaches for modelling biochemical pathways have appeared over the last 30 years, and with the help of computers it is not difficult to simulate behaviour of simple metabolic pathways with a set of equations representing kinetics of the enzymes in the pathway. However, dealing with complex systems and progressing further in the field requires a theoretical basis for formulating different strategies for simplifying the complexity and relating local properties of enzymes with global properties of the pathway.

To fulfil this purpose two approaches to the analysis of complex biochemical systems have emerged in the past two decades, biochemical systems theory [1–3] and metabolic control analysis [4–6]. The need for these derives from the large numbers of components in such systems and the nonlinear interactions between them. Both are based on sensitivity theory, but whereas biochemical systems theory uses explicit methods metabolic control analysis uses implicit methods [7–10]. Equivalent matrix equations have been developed in both formalisms to relate the response of a whole system to a perturbation (logarithmic gains in biochemical systems theory, or control coefficients in metabolic control analysis) and the local responses of individual enzymes to changes in their substrate concentrations (kinetic orders or elasticity coefficients).

Two variants of biochemical systems theory have long been distinguished, generalized mass action and S-systems. In both variants each rate law is simplified by writing it as a product of power-law functions of all metabolites involved. The exponent associated with each metabolite is called a kinetic order and is regarded as a constant. At the operating point it is equivalent to an elasticity coefficient defined in metabolic control analysis, with the difference that in control analysis there is no implication that it is a constant. In generalized mass action each individual reaction is a unit of representation, but in S-systems the individual reaction fluxes are aggregated into net fluxes through each internal metabolite pool of the metabolic pathway; in this way, a steady state is represented simply as the balance between two terms from Kirchhoff's node equations, one representing the aggregation of the incoming fluxes and the other the aggregation of the outgoing fluxes.

The S-system way of representing a system has several advantages [11] but it also has some disadvantages, and several authors have reported that it fails to predict the new steady state correctly when this is far from the operating point in a complex branched metabolic pathway such as the tricarboxylic acid cycle in *Dictyostelium discoideum* [12] or purine metabolism in man [13].

Although metabolic control analysis has often been applied without aggregating elementary fluxes into net fluxes through pools of enzyme-catalysed reactions, such aggregation is not prohibited, and the advantages and disadvantages of aggregation described here also have importance in control analysis if aggregation is used to simplify a complex model. The general strategy used has been to simplify the system into modules or blocks that communicate via one or more explicit intermediates; this approach is called as modular [14,15] or top-down control analysis [16,17]. In contrast to the S-system strategy, the aggregation is accomplished by eliminating intermediates that are included in modules or blocks crossed by the fluxes that become overall fluxes. The S-system strategy do not include the

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Enzymes: hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), ribose-phosphate pyrophosphokinase (EC 2.7.6.1), xanthine dehydrogenase (EC 1.1.1.204), xanthine oxidase (EC 1.1.3.22).

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simplification by eliminating intermediates, but aggregate fluxes around each internal metabolite following different possible combination strategies that must always result in a net synthesis and a net degradation flux through each intermediate pool. The aggregated rate laws are obtained from the values of these fluxes at a given steady state and following the power-law formalism.

Varying the concentration of the linking metabolite and measuring the fluxes through the pathway allows calculation of the kinetic orders [18] or elasticities [19] of the producers and consumers of the linking metabolite with respect to it, regardless of whether this is in the context of S-systems or metabolic control analysis. However, relating the kinetic order or elasticity of an aggregated reaction to the corresponding properties of the component reactions is often difficult.

Aggregation of elementary fluxes into net fluxes of synthesis and degradation through pools, as in S-systems, allows explicit steady-state expressions to be obtained for fluxes and internal metabolite concentrations in terms of the concentrations of external metabolites, cofactors and enzymes. This has been regarded as an advantage of S-systems [3,18] because it permits prediction of the new steady-state fluxes and intermediate concentrations after changes in enzyme activities or other independent variables. However, some authors have reported that the stoichiometry is not necessarily conserved in the predicted steady states [12]. The aggregation may produce a spurious reduction in the number of degrees of freedom of the system at steady state, because the stoichiometric relationships are not accurately expressed by the system equations [20]. Curto *et al*. [13] observed that the stoichiometry of branched systems is destroyed using the S-system formulation for predictions of new steady states when the deviation from the operating point is extensive, and they illustrated the biochemical and clinical importance of these inaccuracies with a model of purine metabolism in man. For example, in modelling deficiency of hypoxanthine phosphoribosyltransferase they found that the inaccuracy in the flux stoichiometry of S-system models became significant for large deviations from the operating point. Torres *et al*. [21] observed similar inaccuracies in the stoichiometry of fluxes with an S-system model for optimizing ethanol, glycerol and carbohydrate production in *Saccharomyces cerevisiae*, and they proposed putting constraints on the values that the fluxes can attain, to minimize the violation of flux stoichiometry and to solve the problem empirically. Despite these experimental reports of stoichiometry violations in S-system models we are not aware of any previous systematic quantitative analysis of the system characteristics that generate the violations.

In this paper we derive appropriate matrix equations that permit conversion in either direction between kinetic orders of rate laws of aggregated reactions and the corresponding kinetic order values of the individual reactions. The same matrix equations also permit an easy interconversion between logarithmic gains or control coefficients for aggregated fluxes and the corresponding parameters for individual fluxes. The equations can be solved both algebraically and numerically, and would be easy to implement in any of the computer programs that are available for dealing with biochemical systems theory or metabolic control analysis [22–27]. Moreover, they not only simplify the task of relating any kinetic properties of an individual reaction with the kinetic properties of aggregated reactions; we also use them to demonstrate the important point that the cause of violation of stoichiometry is the fact that treating aggregated kinetic orders (or aggregated elasticities) as constant means assuming not only that the kinetic orders or elasticities of individual steps are constant but also that the ratios between individual fluxes in branch points are constant from one steady state to another. In addition, we show that exhaustive analysis of the terms of the matrix equations presented in this paper permit prediction of the extent and importance of the stoichiometry violation, and thus evaluation of when aggregating fluxes in the nodes to simplify a model will be appropriate. In fact, as we show, aggregation of elementary fluxes in branched metabolic systems into net fluxes for synthesis and degradation through pools does cause considerable violation of stoichiometric properties, to a degree depending on the extent to which the flux distribution at each branch point in the system changes in response to a perturbation of an enzyme concentration or any other independent parameter of the system. Branched pathways are regulated *in vivo* in such a way that changes in enzyme activities can produce large changes in the distribution of flux around the branch points.

Integrating the control-coefficient functions in logarithmic space leads to the same values of steady-state variables after a perturbation as those predicted with the S-system explicit steady-state solutions for fluxes and internal metabolite concentrations. It follows, therefore, that the same problem of violation of stoichiometric constraints arises in metabolic control analysis if large changes are predicted by assuming constant control coefficients.

Although it has been argued that metabolic control analysis is focussed on the effects of small perturbations around the operating point [28,29], there is increasing emphasis on the predictive value of control coefficients for rational metabolic engineering. In this point of view metabolic engineers may be tempted to predict the consequences of large changes in enzyme activities from the steady-state values of control coefficients assumed as constants, but as we demonstrate in this paper the new steady state predicted will violate flux stoichiometry in the same manner as S-system predictions.

THEORY AND DEFINITIONS

For definition purposes we consider the pathway shown in Fig. 1A, where X_0 is an external metabolite kept at a constant concentration, S_1, S_2 and S_3 are internal metabolites whose steady-state concentrations are determined by the system, E_1–E_5 are the enzymes that catalyse the five steps shown, which have individual rates v_1–v_5, respectively. Here we follow the usual biochemical practice of using distinct symbols for metabolites and enzymes, and the additional practice common in metabolic control analysis of distinguishing symbolically between external and internal metabolites. In biochemical systems theory it is more usual to stress the common characteristic of both enzymes and metabolites in possessing concentrations that influence the properties of the system, and so the formulation shown in Fig. 1B would be closer to the spirit of that approach. To avoid confusion we shall use only the symbolism of Fig. 1A in the remainder of the paper, but it should be evident that Fig. 1A and 1B are identical apart from symbols and that transposition between the two is straightforward. Likewise although we shall also use additional terminology and symbols from metabolic control analysis (control coefficients, elasticities, etc.), there is a one-to-one correspondence with the terminology and symbols of biochemical systems theory (logarithmic gains, kinetic orders, etc.) and transposition is again straightforward (Table 1). We shall mention the latter alternatives in this section.

A flux control coefficient $C^J_{e_i}$ (logarithmic gain) expresses the normalized sensitivity of the flux J through any step of the

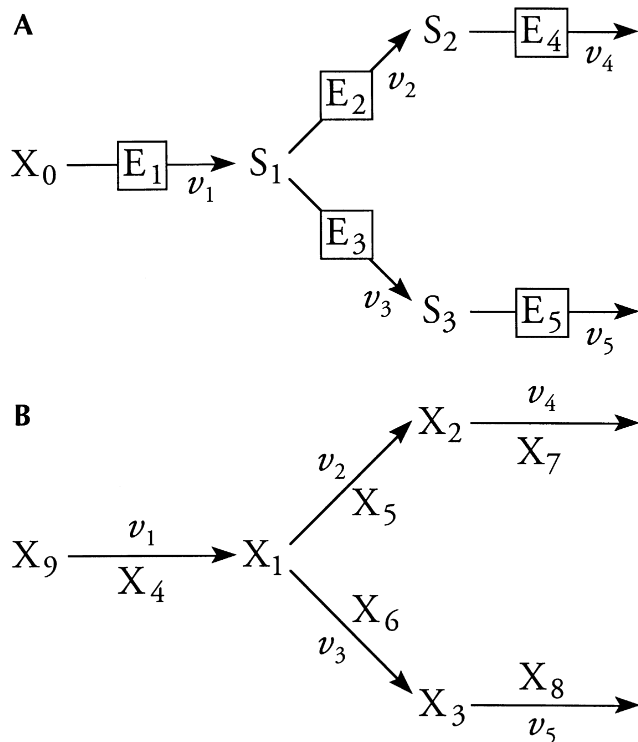

Fig. 1. Model of a branched pathway. In (A) the model is labelled according to common biochemical practice, using E for enzymes, X for an external metabolite and S for internal metabolites. In (B) the same model is labelled in accordance with the practice of biochemical systems theory, with the same symbol X used for both enzymes and metabolites, to emphasize the fact that rates depend on the concentrations of both.

pathway to the concentration e_i of any enzyme E_i (not necessarily the enzyme that catalyses the step in question), defined as follows:

$$C^J_{e_i} = \frac{\partial \ln J}{\partial \ln e_i}$$

or more generally,

$$C^J_{v_i} = \frac{\partial \ln J}{\partial \ln p_i} \bigg/ \frac{\partial \ln v_i}{\partial \ln p_i}$$

where p_i is a parameter that specifically perturbs the rate v_i.

A concentration control coefficient $C^{s_i}_{e_i}$ expresses the same relationship for any metabolite concentration s_j:

$$C^{s_i}_{e_i} = \frac{\partial \ln s_j}{\partial \ln e_i}$$

The partial derivative of the rate v_j of an individual reaction with respect to the concentration s_i of any metabolite S_i, with all other concentrations held constant, is called an elasticity (ε) in metabolic control analysis (kinetic order in biochemical systems theory):

$$\varepsilon^{v_j}_{s_i} = \frac{\partial \ln v_j}{\partial \ln s_i}$$

The partial derivative of the rate v_j of an individual reaction with respect to the concentration e_i of any enzyme E_i, with all other enzyme concentrations held constant, is likewise an elasticity or kinetic order, and can be symbolized in the same sort of way:

$$\varepsilon^{v_j}_{e_i} = \frac{\partial \ln v_j}{\partial \ln e_i}$$

When the enzymes are independent catalysts (i.e. each enzyme rate is strictly proportional to the total enzyme concentration and there are no enzyme–enzyme interactions), enzyme elasticities are exactly 1 for $i = j$ and exactly 0 for $i \neq j$, i.e. the matrix of enzyme elasticities is the identity matrix ($\mathbf{I}_5$). When either of these conditions fails, the matrix of enzyme elasticities takes a less trivial form, and some authors find it useful to refer to them as π-elasticities (with the symbol π instead of ε) to distinguish them from metabolite elasticities; however, they are not conceptually different from other elasticities and it is arguable whether anything is gained by giving them a different name and symbol.

In the context of metabolic control analysis the relationships between control coefficients and elasticities have been expressed in a number of matrix equation formats [7,8,17,30–32]. Concise formulations are those of Westerhoff and Kell [17] derived from the theorems of metabolic control analysis, and those of Cascante *et al.* [7,8] obtained by implicit differentiation of steady-state equations. We will use the latter formulations in this paper.

In the system represented by Fig. 1 it follows from elementary kinetics that the vector of steady-state fluxes through the steps of the pathway $\mathbf{J} = [J_1\ J_2\ J_3\ J_4\ J_5]^T$ is a function of the vector of steady-state internal metabolite concentrations $\mathbf{s} = [s_1\ s_2\ s_3]^T$ and the vector of enzyme concentrations $\mathbf{e} = [e_1\ e_2\ e_3\ e_4\ e_5]^T$, and differentiating the vector of fluxes with respect to the vector of enzyme concentrations and normalizing, we obtain:

$$\left[\frac{\mathrm{d}\ln \mathbf{J}}{\mathrm{d}\ln \mathbf{e}}\right] = \left[\frac{\partial \ln \mathbf{v}}{\partial \ln \mathbf{s}}\right]\left[\frac{\mathrm{d}\ln \mathbf{s}}{\mathrm{d}\ln \mathbf{e}}\right] + \left[\frac{\partial \ln \mathbf{v}}{\partial \ln \mathbf{e}}\right] \quad (1)$$

This equation relates the global steady-state behaviour of the system to its local kinetic properties. Replacing the partial derivative expressions by the corresponding control coefficients and elasticities this takes the following form:

$$\mathbf{C}^{\mathbf{J}}_{\mathbf{e}} = \boldsymbol{\varepsilon}_{\mathbf{s}}\mathbf{C}^{\mathbf{s}}_{\mathbf{e}} + \boldsymbol{\varepsilon}_{\mathbf{e}} \quad (2)$$

where, in the case of the system of Fig. 1:

$$\mathbf{C}^{\mathbf{J}}_{\mathbf{e}} = \begin{bmatrix} C^{J_1}_{e_1} & \cdots & C^{J_1}_{e_5} \\ \vdots & \ddots & \vdots \\ C^{J_5}_{e_1} & \cdots & C^{J_5}_{e_5} \end{bmatrix}, \boldsymbol{\varepsilon}_{\mathbf{s}} = \begin{bmatrix} \varepsilon^1_1 & 0 & 0 \\ \varepsilon^2_1 & \varepsilon^2_2 & 0 \\ \varepsilon^3_1 & 0 & \varepsilon^5_3 \\ 0 & \varepsilon^4_2 & 0 \\ 0 & 0 & \varepsilon^5_3 \end{bmatrix},$$

$$\mathbf{C}^{\mathbf{s}}_{\mathbf{e}} = \begin{bmatrix} C^{s_1}_{e_1} & \cdots & C^{s_1}_{e_5} \\ C^{s_2}_{e_1} & \cdots & C^{s_2}_{e_5} \\ C^{s_3}_{e_1} & \cdots & C^{s_3}_{e_5} \end{bmatrix}, \boldsymbol{\varepsilon}_{\mathbf{e}} = \mathbf{I}_5 = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix}$$

Aggregating steps into blocks

The generalized mass action variant of biochemical systems theory treats each individual reaction as a unit for representa-

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Table 1. Comparison between symbols used in this paper with those used in biochemical systems theory. In general in this paper we follow the practice of metabolic control analysis, but we make no attempt to refer to all variations. For biochemical systems theory we list the most commonly used symbols.

Description	This paper	Biochemical systems theory
External metabolite[a]	X_k	X_i
Internal metabolite[a]	S_j	X_i
Enzyme[a]	E_i	X_i
Concentration of X[b]	x	X
Local rate	v_i	V_i
Steady-state flux	J_i	V_i
Flux around metabolite S_k	$\dot{s}_k$	$\dot{S}_k$
Aggregated flux of synthesis of S_k	$\dot{s}_{k+}$	V_{k+}
Aggregated flux of degradation of S_k	$\dot{s}_{k-}$	V_{k-}
Matrix of normalized derivative of flux with respect to enzyme concentration (or activity)[c]	$\mathbf{C}_\mathbf{e}^\mathbf{J}$	$L(V, X_I)$
Matrix of normalized derivative of concentration with respect to enzyme concentration (or activity)[d]	$\mathbf{C}_\mathbf{e}^\mathbf{s}$	$L(X_D, X_I)$
Matrix of normalized partial derivative of local rate with respect to metabolite concentration[e]	$\boldsymbol{\varepsilon}_\mathbf{s}$	f_D
Matrix of normalized partial derivative of local rate with respect to enzyme concentration[f]	$\boldsymbol{\varepsilon}_\mathbf{e}$	f_I
Matrix of normalized partial derivative of aggregated rate of synthesis of metabolite S_k with respect to metabolite concentration[g]	$\boldsymbol{\varepsilon}_\mathbf{s}^+$	G_D
Matrix of normalized partial derivative of aggregated rate of degradation of metabolite S_k with respect to metabolite concentration[g]	$\boldsymbol{\varepsilon}_\mathbf{s}^-$	H_D
Matrix of normalized partial derivative of aggregated rate of synthesis of metabolite S_k with respect to enzyme concentration[g]	$\boldsymbol{\varepsilon}_\mathbf{e}^+$	G_I
Matrix of normalized partial derivative of aggregated rate of degradation of metabolite S_k with respect to enzyme concentration[g]	$\boldsymbol{\varepsilon}_\mathbf{e}^-$	H_I

[a] In metabolic control analysis there are separate numbering systems for metabolites and enzymes; in biochemical systems theory there is a single numbering system common to both, metabolites increasing from 1 to n and enzymes from $n + 1$ to m. Compare Fig. 1A with 1B. [b] Many systems are in use. Not all authors distinguish between the symbol for the chemical identity of a species (roman capital in this paper) and the symbol for its concentration (lower-case italic in this paper), or distinguish only by using roman for one and italic for the other (a distinction that often passes unnoticed). [c] Flux control coefficient in metabolic control analysis and in this paper; logarithmic gain for flux in biochemical systems theory. [d] Concentration control coefficient in metabolic control analysis and in this paper; logarithmic gain for concentration in biochemical systems theory. [e] Elasticity in metabolic control analysis; kinetic order in biochemical systems theory. [f] Enzyme elasticity in this paper (some authors use the term π-elasticity and symbol π instead of ε); kinetic order in biochemical systems theory. [g] No symbols are in common use in metabolic control analysis.

tion. However, if one treats each internal metabolite S_k as a unit for representation, the value of $\dot{s}_k$ at each time point is the sum of the rates of the individual processes producing or degrading it. Hence an alternative description of the system is as follows:

$$\dot{s}_k = \dot{s}_{k+} - \dot{s}_{k-}$$

in which the different processes affecting $\dot{s}_k$ have been aggregated as two net rates, one of synthesis ($\dot{s}_{k+}$) and one of degradation ($\dot{s}_{k-}$). In the literature of biochemical systems theory the symbols V_{k+} and V_{k-} are used rather than $\dot{s}_{k+}$ and $\dot{s}_{k-}$; however, we prefer $\dot{s}_{k+}$, and $\dot{s}_{k-}$, as they are less likely to encourage confusion with the rates of the individual enzyme-catalysed steps or with their forward and reverse limiting rates. It is also important to make it clear that the indices used refer to metabolites rather than to steps. Representing the pathway of Fig. 1 in this way produces the following set of equations:

$$\dot{s}_1 = v_1 - (v_2 + v_3) = \dot{s}_{1+} - \dot{s}_{1-}$$

$$\dot{s}_2 = v_2 - v_4 = \dot{s}_{2+} - \dot{s}_{2-}$$

$$\dot{s}_3 = v_3 - v_5 = \dot{s}_{3+} - \dot{s}_{3-}$$

Any metabolic scheme can be represented in this way to produce a set of equations known in biochemical systems theory as an S-system.

For the system of Fig. 1, conversion of individual fluxes into aggregated fluxes gives us a set of constraints in the vector of fluxes of individual reactions that can be listed systematically:

$$\dot{s}_{1+} = v_1$$

$$\dot{s}_{2+} = v_2$$

$$\dot{s}_{3+} = v_3$$

$$\dot{s}_{1-} = v_2 + v_3$$

$$\dot{s}_{2-} = v_4$$

$$\dot{s}_{3-} = v_5$$

Flux control coefficients and elasticities can now be defined as follows with respect to aggregated fluxes:

$$C_{e_i}^{\dot{s}_{k+}} = \frac{\mathrm{d}\ln \dot{s}_{k+}}{\mathrm{d}\ln e_i}, C_{e_i}^{\dot{s}_{k-}} = \frac{\mathrm{d}\ln \dot{s}_{k-}}{\mathrm{d}\ln e_i} \quad (3)$$

$$*\varepsilon_{s_i}^{k+} = \frac{\partial \ln \dot{s}_{k+}}{\partial \ln s_i}, *\varepsilon_{s_i}^{k-} = \frac{\partial \ln \dot{s}_{k-}}{\partial \ln s_i} \quad (4)$$

$$*\varepsilon_{e_i}^{k+} = \frac{\partial \ln \dot{s}_{k+}}{\partial \ln e_i}, *\varepsilon_{e_i}^{k-} = \frac{\partial \ln \dot{s}_{k-}}{\partial \ln e_i} \quad (5)$$

Although in a sense the derivatives in Eqn (3) are just as partial as those in Eqns (4) and (5), we prefer to regard them as total derivatives to emphasize that they refer to systemic properties, whereas those in Eqns (4) and (5) refer to local properties. The known relationships between the aggregated rates $\dot{s}_+$ or $\dot{s}_-$ and the individual rates v then allow one to relate these control coefficients and elasticities for the aggregated fluxes to those for the individual steps.

By differentiating the vectors of aggregated fluxes $\dot{\mathbf{s}}_+$ or $\dot{\mathbf{s}}_-$ with respect to the vector of individual reaction fluxes or rates (remembering that $\mathbf{J} = \mathbf{v}$ when the system is at steady state) and normalizing, we obtain the link matrices of aggregated fluxes with respect to individual reactions:

$$\mathbf{L}_{\dot{\mathbf{s}}_+} = \frac{\partial \ln \dot{\mathbf{s}}_+}{\partial \ln \mathbf{v}}$$

$$\mathbf{L}_{\dot{\mathbf{s}}_-} = \frac{\partial \ln \dot{\mathbf{s}}_-}{\partial \ln \mathbf{v}}$$

in general, or in the specific case of the system of Fig. 1:

$$\mathbf{L}_{\dot{\mathbf{s}}_+} = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 \end{bmatrix}$$

$$\mathbf{L}_{\dot{\mathbf{s}}_-} = \begin{bmatrix} 0 & v_2/\dot{s}_{1-} & v_3/\dot{s}_{1-} & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix}$$

Multiplying the left- and right-hand sides of Eqn (1) or (2) by $\mathbf{L}_{\dot{\mathbf{s}}_+}$ or $\mathbf{L}_{\dot{\mathbf{s}}_-}$, we obtain:

$$\mathbf{L}_{\dot{\mathbf{s}}_+}\mathbf{C}_\mathbf{e}^\mathbf{J} = \mathbf{L}_{\dot{\mathbf{s}}_+}\boldsymbol{\varepsilon}_\mathbf{s}\mathbf{C}_\mathbf{e}^\mathbf{s} + \mathbf{L}_{\dot{\mathbf{s}}_+}\boldsymbol{\varepsilon}_\mathbf{e}$$

$$\mathbf{L}_{\dot{\mathbf{s}}_-}\mathbf{C}_\mathbf{e}^\mathbf{J} = \mathbf{L}_{\dot{\mathbf{s}}_-}\boldsymbol{\varepsilon}_\mathbf{s}\mathbf{C}_\mathbf{e}^\mathbf{s} + \mathbf{L}_{\dot{\mathbf{s}}_-}\boldsymbol{\varepsilon}_\mathbf{e}$$

According to the above definitions we can then write the following relationships between the aggregated flux control coefficients and those for the individual reactions:

$$\mathbf{L}_{\dot{\mathbf{s}}_+}\mathbf{C}_\mathbf{e}^\mathbf{J} = \mathbf{C}_\mathbf{e}^{\dot{\mathbf{s}}_+} \quad (6)$$

$$\mathbf{L}_{\dot{\mathbf{s}}_-}\mathbf{C}_\mathbf{e}^\mathbf{J} = \mathbf{C}_\mathbf{e}^{\dot{\mathbf{s}}_-} \quad (7)$$

The relationship between aggregated elasticities and elasticities for individual reactions follows in a similar manner:

$$\mathbf{L}_{\dot{\mathbf{s}}_+}\boldsymbol{\varepsilon}_\mathbf{s} = \frac{\partial \ln \dot{\mathbf{s}}_+}{\partial \ln \mathbf{s}} = *\boldsymbol{\varepsilon}_\mathbf{s}^+ \quad (8)$$

$$\mathbf{L}_{\dot{\mathbf{s}}_-}\boldsymbol{\varepsilon}_\mathbf{s} = \frac{\partial \ln \dot{\mathbf{s}}_-}{\partial \ln \mathbf{s}} = *\boldsymbol{\varepsilon}_\mathbf{s}^- \quad (9)$$

$$\mathbf{L}_{\dot{\mathbf{s}}_+}\boldsymbol{\varepsilon}_\mathbf{e} = \frac{\partial \ln \dot{\mathbf{s}}_+}{\partial \ln \mathbf{e}} = *\boldsymbol{\varepsilon}_\mathbf{e}^+ \quad (10)$$

$$\mathbf{L}_{\dot{\mathbf{s}}_-}\boldsymbol{\varepsilon}_\mathbf{e} = \frac{\partial \ln \dot{\mathbf{s}}_-}{\partial \ln \mathbf{e}} = *\boldsymbol{\varepsilon}_\mathbf{e}^- \quad (11)$$

Aggregation of fluxes through each node in a net flux of synthesis and a net flux of degradation has the advantage that the steady-state equations can be solved explicitly for each dependent variable from the fundamental equations governing the behaviour of the intact biochemical system, and in S-systems the explicit solutions for the steady state have long been known [18,33], and the flux through any pool S_i in the steady state may be obtained by a simple secondary calculation involving the known steady-state concentrations [34]. The resulting explicit solutions give the complete relationship in S-systems between the steady-state values of the dependent variables on the one hand and the values of the independent variables and parameters of the system on the other.

The explicit steady-state equations for internal metabolites and fluxes allow prediction of the new steady state reached when an enzyme concentration (or its activity) is changed. However, the calculation implicitly assumes that the aggregated elasticities have the same values at the initial and final steady state, as they are treated as constants when solving the equations. This not only means that each individual rate equation can be accurately approximated by a power law but also that $\mathbf{L}_{\dot{\mathbf{s}}_+}$ and $\mathbf{L}_{\dot{\mathbf{s}}_-}$ are also equal in the two steady states. The first assumption may sometimes be correct, as many enzymes work well below saturation and the logarithm of the rate is then almost a linear function of the logarithm of its substrate concentration over a wide range of concentrations. However the second assumption cannot usually be true in general for a branched system, as it is common for such systems that the partitioning of flux between branches is highly sensitive to changes in enzyme activities. So, as the individual elements of $\mathbf{L}_{\dot{\mathbf{s}}_+}$ and $\mathbf{L}_{\dot{\mathbf{s}}_-}$ are 0, 1 or ratios, they can change dramatically from one steady state to another, and very unrealistic predictions of the new steady state can result.

Moreover, the new steady-state fluxes predicted with an S-system can violate the flux stoichiometry at each branch point, because the constraints on individual fluxes are not taken into account in the explicit steady-state equations, the fluxes being considered independent. In the specific case considered $\dot{s}_{2+}$ and $\dot{s}_{3+}$ are dependent on v_1 and this is not taken in account in obtaining the explicit solutions.

A new steady state can also be predicted directly from control coefficient values considered as constants. Thus, from the general definition of a control coefficient as

$$C_{e_i}^Y = \frac{\mathrm{d}\ln Y}{\mathrm{d}\ln e_i}$$

therefore, we can integrate from the reference state e_i to a new state $e_i + \Delta e_i$ to obtain a new steady-state value of any systemic variable Y:

$$\ln Y(e_i + \Delta e_i) = \ln Y(e_i) + C_{e_i}^Y[\ln(e_i + \Delta e_i) - \ln(e_i)]$$

This calculation assumes, however, that the $C_{e_i}^Y$ are constants from one steady state to another, and recognizing that

$$\begin{bmatrix} \mathbf{C}_\mathbf{e}^\mathbf{J} \\ \cdots \\ \mathbf{C}_\mathbf{e}^\mathbf{s} \end{bmatrix} = \begin{bmatrix} \dfrac{\mathrm{d}\ln \mathbf{J}}{\mathrm{d}\ln \mathbf{J_I}} & \vdots & \boldsymbol{\varepsilon}_\mathbf{s} \end{bmatrix}^{-1}$$

where $\mathbf{J_I}$ are the independent fluxes of the system, it is easy to realize that as $\mathrm{d}\ln\mathbf{J}/\mathrm{d}\ln\mathbf{J_I}$ is a matrix of flux ratios, we also are assuming here that the ratios between fluxes do not change from one steady state to another. So, for a branched system calculating a new steady state after a finite perturbation by assuming constancy of control coefficients could violate the flux stoichiometry of the system.

We now illustrate how flux stoichiometry can be violated by reference to a specific numerical example based on the system of Fig. 1, deliberately choosing the simplest example in which violation of stoichiometry is possible. With a more complex pathway and with reversibility of the reactions, the problem can occur more severely, as we shall describe in detail in the Discussion section for the case of enzyme deficiencies in an S-system model of purine metabolism in man. The individual rate equations and steady-state values of the system variables are as follows in the reference steady state:

$$\dot{s}_1 = v_1 - v_2 - v_3, \dot{s}_2 = v_2 - v_4, \dot{s}_3 = v_3 - v_5$$

where

$$\ln v_1 = \ln \gamma_1 + \varepsilon^{v_1}_{x_0} \ln x_0 + \varepsilon^{v_1}_{e_1} \ln e_1; v_1 = 0.32 x_0^{0.5} e_1$$

$$\ln v_2 = \ln \gamma_2 + \varepsilon^{v_2}_{s_1} \ln s_1 + \varepsilon^{v_2}_{e_2} \ln e_2; v_2 = 0.02 s_1^{0.8} e_2$$

$$\ln v_3 = \ln \gamma_3 + \varepsilon^{v_3}_{s_1} \ln s_1 + \varepsilon^{v_3}_{e_3} \ln e_3; v_3 = 0.08 s_1^{0.2} e_3$$

$$\ln v_4 = \ln \gamma_4 + \varepsilon^{v_4}_{s_2} \ln s_2 + \varepsilon^{v_4}_{e_4} \ln e_4; v_4 = 0.18 s_2^{0.5} e_4$$

$$\ln v_5 = \ln \gamma_5 + \varepsilon^{v_5}_{s_3} \ln s_3 + \varepsilon^{v_5}_{e_5} \ln e_5; v_5 = 0.04 s_3^{0.5} e_5$$

and

$$\varepsilon^{v_1}_{x_0} = 0.5, \varepsilon^{v_2}_{s_1} = 0.8, \varepsilon^{v_3}_{s_1} = 0.2, \varepsilon^{v_4}_{s_2} = 0.5, \varepsilon^{v_5}_{s_3} = 0.5,$$

$$\varepsilon^{v_1}_{e_1} = \varepsilon^{v_2}_{e_2} = \varepsilon^{v_3}_{e_3} = \varepsilon^{v_4}_{e_4} = \varepsilon^{v_5}_{e_5} = 1,$$

$$e_1 = e_2 = e_3 = e_4 = e_5 = 10,$$

$$x_0 = 10$$

The algebraic forms of these equations are written in terms of logarithms to avoid having illegible superscript indices, but one may readily verify that they are equivalent to the numerical versions shown on the right if $\gamma_1 = 0.32$, $\gamma_2 = 0.02$, $\gamma_3 = 0.08$, $\gamma_4 = 0.18$, $\gamma_5 = 0.04$.

Aggregating fluxes now for net synthesis and degradation of each metabolite, we have

$$\dot{s}_1 = \dot{s}_{1+} - \dot{s}_{1-}, \dot{s}_2 = \dot{s}_{2+} - \dot{s}_{2-}, \dot{s}_3 = \dot{s}_{3+} - \dot{s}_{3-}$$

where

$$\dot{s}_{1+} = v_1 = 0.32 x_0^{0.5} e_1$$

$$\dot{s}_{1-} = v_2 + v_3 = 0.02 s_1^{0.8} e_2 + 0.08 s_1^{0.2} e_3$$

$$\dot{s}_{2+} = v_2 = 0.02 s_1^{0.8} e_2$$

$$\dot{s}_{2-} = v_4 = 0.18 s_2^{0.5} e_4$$

$$\dot{s}_{3+} = v_3 = 0.08 s_1^{0.2} e_3$$

$$\dot{s}_{3-} = v_5 = 0.04 s_3^{0.5} e_5$$

Five of these equations are usable as they stand for predicting the new steady state that results from a perturbation by solving the explicit system, but the second equation, the expression for $\dot{s}_{1-}$, needs to be modified to avoid the sum of two terms on the right-hand side. Following standard methods from biochemical systems theory, we can re-express this equation as follows:

$$\dot{s}_{1-} = \beta_1 s_1^{*\varepsilon^{\dot{s}_{1-}}_{s_1}} e_2^{*\varepsilon^{\dot{s}_{1-}}_{e_2}} e_3^{*\varepsilon^{\dot{s}_{1-}}_{e_3}} = 0.04 s_1^{0.68} e_2^{0.8} e_3^{0.2}$$

where

$$*\varepsilon^{\dot{s}_{1-}}_{s_1} = \frac{\varepsilon^{v_2}_{s_1} v_2 + \varepsilon^{v_3}_{s_1} v_3}{v_2 + v_3} = 0.68$$

$$*\varepsilon^{\dot{s}_{1-}}_{e_2} = \frac{\varepsilon^{v_2}_{e_2} v_2}{v_2 + v_3} = 0.8$$

$$*\varepsilon^{\dot{s}_{1-}}_{e_3} = \frac{\varepsilon^{v_3}_{e_3} v_3}{v_2 + v_3} = 0.2$$

$$\beta_1 = \frac{v_2 + v_3}{s_1^{*\varepsilon^{\dot{s}_{1-}}_{s_1}} e_2^{*\varepsilon^{\dot{s}_{1-}}_{e_2}} e_3^{*\varepsilon^{\dot{s}_{1-}}_{e_3}}} = 0.04$$

This re-expression is designed to give the correct derivatives at the reference state, but there is no guarantee that the linear model that can be constructed from it will give correct values away from the reference state, because there is no guarantee that the coefficients remain constant. As shown in Table 2, the new steady states obtained from the model when any enzyme concentration is decreased fivefold give results in which $\dot{s}_{1-} \neq \dot{s}_{2+} + \dot{s}_{3+}$, violating the stoichiometric requirements for the system. Notice that although the net fluxes and metabolite concentrations do not differ by large factors from the values given by generalized mass action, the discrepancies in the stoichiometries are not negligible: for example in the second line $\dot{s}_{2+} + \dot{s}_{3+}$ is 25% larger than $\dot{s}_{1-}$, which means that the pathway is producing 25% more end product than the amount of starting material consumed.

Using parameters for aggregated reactions to calculate those for the individual reactions

Let us consider the characterization of a branched pathway using the S-system strategy and let us assume that we know the matrices of control coefficients with respect to aggregated fluxes and of aggregated elasticities.

In this case, the question arises of whether it is always possible to obtain control coefficients or elasticities for the individual reactions from the control coefficients and elasticities of the aggregated reactions? We will show in this section that it is not always possible, and we will give practical rules to predict whether a given aggregation strategy allows us to lose information about individual reactions.

Converting aggregated to individual flux control coefficients

We have seen above that the set of relationships between aggregated control coefficients ($\mathbf{C}^{\dot{\mathbf{s}}_+}_{\mathbf{e}}$ or $\mathbf{C}^{\dot{\mathbf{s}}_-}_{\mathbf{e}}$) and individual reaction control coefficients ($\mathbf{C}^{\mathbf{J}}_{\mathbf{e}}$) are expressed in Eqns (6) and (7).

To obtain the values of $\mathbf{C}^{\mathbf{J}}_{\mathbf{e}}$ as a function of $\mathbf{C}^{\dot{\mathbf{s}}_+}_{\mathbf{e}}$ and $\mathbf{C}^{\dot{\mathbf{s}}_-}_{\mathbf{e}}$ using Cramer's rule to solve the system of equations, the necessary and sufficient condition is that the rank of the matrix of coefficients for aggregated fluxes is equal to the rank of the matrix of coefficients for nonaggregated fluxes:

$$\mathrm{rank}\begin{pmatrix} \mathbf{C}^{\dot{\mathbf{s}}_+}_{\mathbf{e}} \\ \cdots \\ \mathbf{C}^{\dot{\mathbf{s}}_-}_{\mathbf{e}} \end{pmatrix} = \mathrm{rank}\left(\mathbf{C}^{\mathbf{J}}_{\mathbf{e}}\right)$$

However, if

Table 2. Violation of stoichiometric constraints in models obtained with the S-system approach. The top line of the table shows the unperturbed model, i.e. the reference state from which the S-system model was calculated. The other lines show the values obtained when the parameters indicated in the left-hand column were decreased by a factor of five, i.e. in each case from 10.0 to 2.0. The columns labelled Generalized mass action and S-system show the new steady states predicted by two different models, the former treating the individual steps as units of representation, the latter using aggregated fluxes at the branch-point metabolite. As seen in the column for $\dot{s}_{1-} - \dot{s}_{2+} - \dot{s}_{3+}$, which would show a total of 0.0 in every line if the stoichiometric constraints were obeyed, the S-system model leads to violation of stoichiometry around the branch point, i.e. in every case the total flux out of S_1 exceeds the flux in.

	Generalized mass action						S-system						
Perturbation	v_1	$v_2 = v_4$	$v_3 = v_5$	s_1	s_2	s_3	$\dot{s}_{1+} = \dot{s}_{1-}$	$\dot{s}_{2+} = \dot{s}_{2-}$	$\dot{s}_{3+} = \dot{s}_{3-}$	$\dot{s}_{1-} - \dot{s}_{2+} - \dot{s}_{3+}$	s_1	s_2	s_3
None	10.0	8.0	2.0	100.0	20.0	30.0	10.0	8.0	2.0	0.0	100.0	20.0	30.0
e_1	2.0	0.9	1.1	6.1	0.2	9.8	2.0	1.2	1.2	–0.4	9.4	0.5	11.6
e_2	10.0	7.1	2.9	643.8	15.7	63.2	10.0	7.3	2.9	–0.2	664.2	16.6	64.0
e_3	10.0	9.6	0.4	125.3	28.7	1.3	10.0	11.7	0.4	–2.1	160.5	42.7	1.5
x_0	4.5	2.9	1.6	28.3	2.7	18.1	4.5	3.1	1.6	–0.2	30.6	3.0	18.7

$$\operatorname{rank}\begin{pmatrix} \mathbf{C}_{\mathbf{e}}^{\dot{\mathbf{s}}_+} \\ \cdots \\ \mathbf{C}_{\mathbf{e}}^{\dot{\mathbf{s}}_-} \end{pmatrix} < \operatorname{rank}\left(\mathbf{C}_{\mathbf{e}}^{\mathbf{J}}\right)$$

then it is not possible to obtain the complete set of individual flux control coefficients from the set of aggregated flux control coefficients.

For the pathway of Fig. 1 according to the S-system equations obtained with any possible aggregation strategy we always obtain

$$\operatorname{rank}\left(\mathbf{C}_{\mathbf{e}}^{\dot{\mathbf{s}}_{+(-)}}\right) = \operatorname{rank}\left(\mathbf{C}_{\mathbf{e}}^{\mathbf{J}}\right) = 2$$

Thus in this case we can obtain all the individual flux control coefficients from those for aggregated fluxes. However in more complex branched systems it may be possible to lose control information for some individual processes if an inappropriate aggregation strategy is used.

Converting aggregated to individual elasticities

Analogously, if we consider Eqns. (8–11), it is easy to see that to obtain the values of individual reaction elasticities from aggregated elasticities using Cramer's rule the necessary and sufficient condition is:

$$\operatorname{rank}\begin{pmatrix} {}^*\varepsilon_{\mathbf{s}}^{+} \\ \cdots \\ {}^*\varepsilon_{\mathbf{s}}^{-} \end{pmatrix} = \operatorname{rank}(\varepsilon_{\mathbf{s}})$$

$$\operatorname{rank}\begin{pmatrix} {}^*\varepsilon_{\mathbf{e}}^{+} \\ \cdots \\ {}^*\varepsilon_{\mathbf{e}}^{-} \end{pmatrix} = \operatorname{rank}(\varepsilon_{\mathbf{e}})$$

In the example of Fig. 1, the same analysis already done for the control coefficients applies also to the elasticities.

DISCUSSION

In this paper we have derived appropriate matrix equations that clearly show that the aggregated kinetic orders or elasticities are functions of two separate sets of data that can be arranged in two matrices, the matrix of individual kinetic orders or elasticities, and the link matrix of the aggregated fluxes, whose terms are ratios between individual fluxes at each branch point. In addition we have demonstrated that the extension and importance of this violation of stoichiometry can be predicted from an exhaustive analysis of this link matrix.

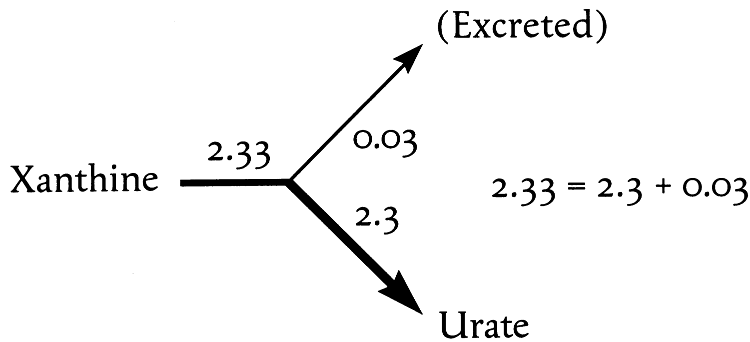
Wright and Field [12] suggested on the basis of computer simulation that the S-system approach could result in the violation of stoichiometric constraints but their suggestion has not previously been studied mathematically. Torres *et al*. [21] described the risk of inaccuracies in the stoichiometry of fluxes as a general characteristic of S-system representations. They suggested the introduction of constraints in the permitted values of fluxes when modelling biochemical systems with S-systems, the necessary constraints being determined empirically for each system.

Curto *et al*. [13] observed in a model of purine metabolism in man that the stoichiometry of branched systems is destroyed using the S-system formulation for predictions of new steady states when the deviation from the operating point is extensive. Xanthine occurs at a branch point, and can be degraded by either of two fluxes: it can be converted into urate, in a reaction catalysed by xanthine oxidase or xanthine dehydrogenase, with a flux of 2.3 μmol·min^{-1}; alternatively it can be excreted, with a flux of 0.03 μmol·min^{-1}. Here and below, fluxes are given per of kg body weight. In the S-system model with aggregation of fluxes the net flux of degradation of xanthine is 2.3 + 0.03 μmol·min^{-1} (Fig. 2A). When the model is altered these fluxes change and there is significant inaccuracy in the flux stoichiometry of the S-system model. For example, for deficiency of hypoxanthine phosphoribosyltransferase the individual calculated fluxes are 14.0 and 21.5 μmol·min^{-1}, respectively, but the combined flux, which ought to be the sum of these two values, is only 15.1 μmol·min^{-1} (Fig. 2B). In addition, a large and unphysiological accumulation of xanthine and hypoxanthine is predicted by the S-system model for this deficiency.

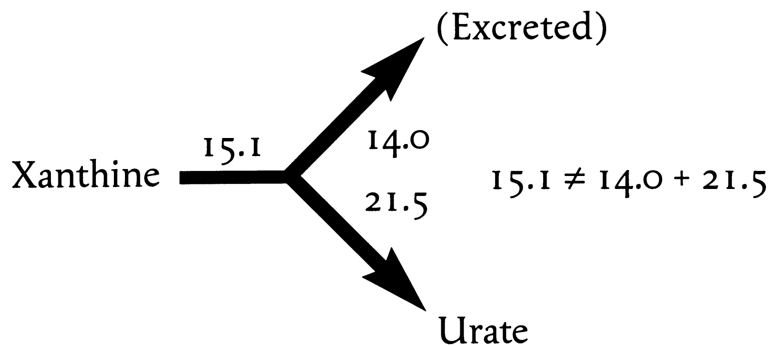
By contrast, excessive activity of ribose-phosphate pyrophosphokinase leads to less anomalous values of 4.3 and 0.3 μmol·min^{-1} for the individual fluxes and 4.4 μmol·min^{-1} for the combined flux (Fig. 2C).

Expressing the elasticities for aggregated rate laws in terms of the newly defined link matrix of aggregated fluxes, allows quantitative analysis of whether the new steady states estimated with an S-system model significantly violate the stoichiometric flux constraints. This is potentially a major source of error, and to minimize it we conclude that it may be advisable to check

A *Normal*



B *Hypoxanthine phosphoribosyltransferase deficiency*



C *Excess ribose-phosphate pyrophosphokinase*

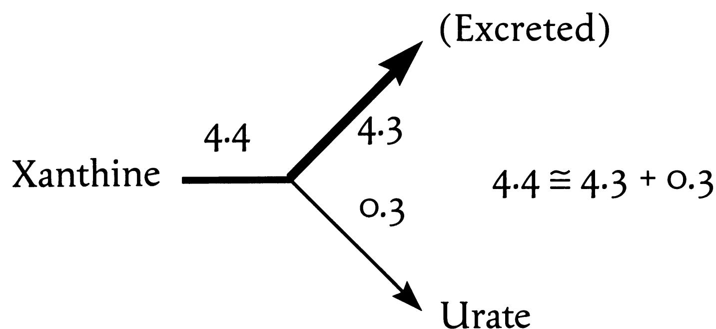


Fig. 2. Stoichiometry violation in models of purine metabolism. (A) The operating point is taken as the state calculated for a normal individual, and in this case the combined flux for degradation of xanthine is the sum of the individual fluxes. (B) However, prediction of effects of deficiency of hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) gives an impossible state in which the combined flux is very different from the sum of the individual fluxes. (C) By contrast, the same sort of calculation for excess ribose-phosphate pyrophosphokinase gives a state where the violation of stoichiometry is negligible.

the sensitivities of the aggregated elasticities to changes in the terms of the $\mathbf{L}_{\dot{S}_+}$ and $\mathbf{L}_{\dot{S}_-}$ matrices before using the S-system representation to model a metabolic system. The matrix equations proposed here to analyse this problem indicate that the deviation of flux stoichiometry will be more pronounced when the system is more sensitive to changes in the flux distribution ratio between the two branches in response to the perturbation. Accordingly, if we analyse the previous example of the S-system model of purine metabolism in man [13] we see that the ratio of fluxes in the two branches is 2.3/0.03 = 76.7 in the reference steady state. With deficiency in hypoxanthine phosphoribosyltransferase this ratio changes about 100-fold to 14.0/21.5 = 0.65, and the consequent violation of stoichiometry is very important, whereas it only decreases by a factor of five in the case of excessive activity of ribose-phosphate pyrophosphokinase, and the violation of stoichiometry is moderate.

This is not, of course, a problem specific to S-systems. Although it is well known in metabolic control analysis that control coefficient values may change from one operating point to another, examples exist where values change little over quite a large range of enzyme concentrations, and one may be tempted to predict new steady states directly by treating them as constants. However, prediction of a new steady state from control coefficients computed in one steady state and treated as constants can result in violation of stoichiometry even if there is

no aggregation of fluxes at branch points. It is especially important to be take this into account when metabolic control analysis is used in rational metabolic engineering to predict directly the new steady state of the pathway after a change in an enzyme activity.

Finally, we have described a test of when it is possible to obtain information about individual reactions from the aggregated reactions and to assess when the aggregation results in loss of information. Using the aggregation matrix proposed in this paper we can systematically and easily analyse different strategies of aggregation to determine which will result in the best representation of the system under study. The equations proposed can easily be implemented in any software package developed for dealing with metabolic control analysis or biochemical systems theory to analyse when predictions are likely to violate stoichiometric constraints.

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