Structure identifiability in metabolic pathways: parameter estimation in models based on the power-law formalism

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An important step in understanding a metabolic pathway is to identify its structure, in terms of the flow of material and information. In pursuing this goal, the available information for a given system is usually obtained from experiments *in vitro* and comes from different sources. Frequently, the final set of regulatory signals acting in the system *in vivo* is unclear, and some kind of test is needed on the intact system. Besides defining an appropriate experimental approach, identification of the regulatory pattern needs a theoretical framework in which the different experimental measurements can be evaluated and a final picture can be agreed on. Mathematical approaches based on sensitivity coefficients provide a useful tool for addressing this problem. Within this framework, the appropriate parameters are related to both the structure of the reaction network and the signals that regulate the target system. Thus the identification of the regulatory structure can be related to the estimation of the appropriate set of parameters. In pursuing this goal, we will show the limitations of using steady-state measurements and the usefulness of using dynamic data. We suggest a way to test the regulatory pattern in a given metabolic pathway by combining both kinds of data, and we show, by using a reference system, the potential of the method suggested.

INTRODUCTION

Metabolic pathways are characterized by components with non-linear behaviour (enzyme reactions and transport systems), interconnected by a high number of regulatory signals, which are ultimately responsible for the co-ordinated behaviour of the system. In considering the structure of a metabolic pathway, we can separate two factors contributing to an observable behaviour in a given condition: (1) the flow of material and (2) the flow of information. In many cases, although the diagram of the reactions responsible for the flow of material through the system is well established, the regulatory pattern, which corresponds to the set of different signals responsible for the flow of information, offers several alternatives that need to be tested. These alternatives, in general, come after considering the data obtained from experiments *in vitro* and from measurements in different conditions. Although this information is valuable, it is now evident that extrapolation of these data to conditions *in vivo* can lead to an inaccurate description of the system [see for instance Shiraishi and Savageau (1992a,b,c,d)]. Hence, a systematic approach is needed so that the system structure can be properly tested from measurements on the intact system.

The complexity shown by a metabolic pathway requires the use of tools specifically devised for investigating the properties of such systems. Here, mathematical models have a decisive role. The mathematical models based on the S-system equations within the Biochemical Systems Theory (BST) specifically represent a given metabolic pathway and can be used to investigate its regulatory structure and properties (Irvine and Savageau, 1985a,b; Savageau, 1972, 1975, 1976, 1979, 1991, 1992; Sorribas and Savageau, 1989a,b; Voit and Savageau, 1982, 1987). A recent review of S-system-related methods can be found in Voit (1991) and references therein. Alternatively, the tools furnished by the Metabolic Control Analysis (MCA) also provide a way of addressing these kinds of problems [see for instance Delgado and Liao (1992a,b) and Sen (1991)].

In a given metabolic system, the existence of regulatory signals determines modulation of the affected reactions. Consequently, the local properties of these reactions will be dependent on these regulatory influences. In both BST and MCA, the local properties of a given process are indicated by appropriate parameters. Within BST, these parameters are g_{ij} and h_{ij}, and they are called kinetic orders:

$$g_{ij} = \left(\frac{\delta V_i^+}{\delta X_j}\right)_0 \frac{X_{j_0}}{V_{i_0}^+}$$

$$h_{ij} = \left(\frac{\delta V_i^-}{\delta X_j}\right)_0 \frac{X_{j_0}}{V_{i_0}^-} \qquad (1)$$

where V_i^+ and V_i^- represent the net processes of synthesis and degradation of X_i [see Sorribas and Savageau (1989a,b,c) and Voit and Savageau (1987) for examples]. The subscript $_0$ indicates evaluation at a given operating point that corresponds to the steady state of interest (Savageau, 1972, 1976; Savageau et al., 1987a,b; Sorribas and Savageau, 1989a,b). In MCA, these parameters are known as elasticity coefficients. It should be stressed that kinetic orders are conceptually equivalent to the elasticity coefficients. The only difference is that kinetic orders are defined for aggregated fluxes and elasticity coefficients are defined for individual fluxes through a given reaction. Translation from one definition to the other requires that the aggregation procedure and the steady-state values of the considered fluxes be taken into account (Savageau et al., 1987a,b; Sorribas and Savageau, 1989a,b).

The kinetic-order parameters (elasticity coefficients) relate to both the structure of the reaction network and the regulatory signals in such a network. For instance, when a reaction is almost saturated by its substrate, the corresponding kinetic order is low. Further, if, in a given pathway, X_5 is an inhibitor of the synthesis of X_3, then $g_{35} < 0$. According to this interpretation, whenever we use BST or MCA, a primary goal for characterizing a given metabolic pathway will be to estimate these parameters from the

Abbreviations used: BST, Biochemical Systems Theory; MCA, Metabolic Control Analysis.

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observed behaviour of the target system. An important point is to identify the set of parameters that is different from zero, i.e. to identify the meaningful influences within the system.

Several procedures based on steady-state measurements have been devised for estimating these kinds of parameter either by direct measurements on the intact system (Savageau, 1976; Savageau et al., 1987a,b; Voit et al., 1991) or, in the case of elasticities and control coefficients, by different experimental procedures that involve experimental modification of the system (Groen, 1984; Groen et al., 1982a,b; Kacser and Burns, 1979; Torres and Meléndez-Hevia, 1991; Torres et al., 1986, 1988; Wanders et al., 1983). Furthermore, different solutions have been suggested for the estimation problem using dynamic data (Johnson, 1988, 1991; Torsella and Bin Razali, 1991; Voit and Savageau, 1982), although rather accurate measurements and initial guesses of the parameter values are required to obtain good estimates (Torsella and Bin Razali, 1991; Voit and Savageau, 1982). In many experimental situations, however, measurements are restricted to initial changes, which may lead to ill-conditioned data and limit the application of the preceding methods (Torsella and Bin Razali, 1991).

All these approaches apply only when both the flow of material and information is well established, that is when the set of g_{ij} and h_{ij} different from zero is known. No strategy has been devised for identifying the structure of the system when it is unknown. In this paper we develop a general approach for addressing this problem. In doing so, we have the following starting points: (1) the flow of material is known; (2) the steady-state fluxes and metabolite concentrations can be measured; (3) the steady state can be manipulated by changing external variables; (4) the transient behaviour of the internal metabolites (or at least of some of them) can be measured after perturbation.

These kinds of measurement can be performed nowadays by a number of techniques and provide valuable information for solving the regulatory structure of the target system. However, we know of no real system in which these kinds of data have been measured. The lack of a theoretical framework for investigating the utility of these data is probably the reason for this. The method developed in this paper will provide a rationale for considering these experiments recommendable.

THEORETICAL RESULTS

Steady-state measurements and identifiability of the regulatory pattern

The steady-state behaviour of a system can be characterized by measuring the changes in the internal variables (i.e. dependent variables) after a change in any of the external variables (i.e. independent variables) and parameters of the system. These measurements correspond to Logarithmic Gains and parameter sensitivities in BST, and to Control and Response Coefficients in MCA. Again, as in the case of the local parameters, translation is immediate from one nomenclature to the other because conceptually they are referring to the same idea. A Logarithmic Gain (Response Coefficient in MCA if the perturbed variable is an external modifier, Control Coefficient if the perturbed variable is an enzyme) is defined as (Savageau, 1972, 1976; Savageau et al., 1987a,b; Sorribas and Savageau, 1989a; Kacser and Burns, 1973; Burns et al., 1985):

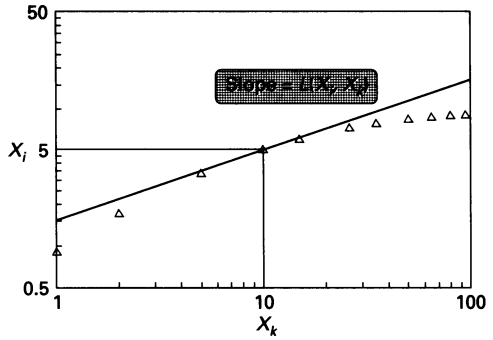

Figure 1. Determination of a Logarithmic Gain from different steady-state measurements

The Logarithmic Gain can be obtained by measuring different steady-state levels of the dependent variable X_i at different values of the independent variable X_k. The slope of the steady-state curve (in logarithmic co-ordinates) at the operating point gives us the value of $L(X_i, X_k)$.

$$L(X_i, X_k) = C^{X_i}_{X_k} = \left(\frac{dX_i}{dX_k}\right)_0 \frac{X_{k_0}}{X_{i_0}} \tag{2}$$

where X_i is a dependent variable ($i = 1, \ldots, n$) and X_k is an independent variable [$k = (n+1), \ldots, (n+m)$]. Similarly, we define the Logarithmic Gain of a given flux as $L(V_i, X_k)$. Logarithmic Gains (control and response coefficients) can be obtained from experiments in which an external variable is perturbed and the system is forced to attain a new steady state (Figure 1). Alternative methods have been described for Logarithmic-Gain (Control and Response Coefficients) measurement in biochemical systems by either direct measurement or appropriate modifications to the pathway [see for instance Groen (1984) and Torres and Meléndez-Hevia. (1991) and references therein]. Recently, alternative procedures have been defined for computing some of these Gains from transient data (Delgado and Liao, 1992a,b).

The set of Logarithmic Gains (i.e. all the Flux Control and Response and all the Concentration Control and Response coefficients) are related to the local parameters (kinetic orders/elasticity coefficients), by the following matrix equation (Savageau and Sorribas, 1989):

$$\mathbf{L}(\mathbf{V_D}, \mathbf{X_I}) = \mathbf{G_I} + \mathbf{G_D}\,\mathbf{L}(\mathbf{X_D}, \mathbf{X_I}) \tag{3}$$

in which **D** refers to dependent variables and **I** refers to independent variables. A similar equation can be written for the MCA approach [see Cascante et al (1989a,b) for details). This equation has a unique solution if the matrices $\mathbf{G_D}$ and $\mathbf{G_I}$ have a definite structure of non-zero elements. Otherwise, for a given set of Logarithmic Gains measured, we can find different $\mathbf{G_D}$ and $\mathbf{G_I}$ matrices that are solution of eqn. (3). Each of those matrices will have a different structure of zero elements. This fact has important implications for the problem of identifying the actual structure of the regulatory signals in our target system.

To appreciate more fully the meaning of eqn. (3), let us consider the case in which $n = 5$ and $m = 3$. Introducing the notation $\mathbf{L}(\mathbf{X_i}, \mathbf{X_k}) = \mathbf{L_{ik}}$ and $\mathbf{L}(\mathbf{V_i^+}, \mathbf{X_k}) = \mathbf{LV_{ik}}$, eqn. (3) is:

$$\begin{bmatrix} LV_{16} & LV_{17} & LV_{18} \\ LV_{26} & LV_{27} & LV_{28} \\ LV_{36} & LV_{37} & LV_{38} \\ LV_{46} & LV_{47} & LV_{48} \\ LV_{56} & LV_{57} & LV_{58} \end{bmatrix} = \begin{bmatrix} g_{16} & g_{17} & g_{18} \\ g_{26} & g_{27} & g_{28} \\ g_{36} & g_{37} & g_{38} \\ g_{46} & g_{47} & g_{48} \\ g_{56} & g_{57} & g_{58} \end{bmatrix} + \begin{bmatrix} g_{11} & g_{12} & g_{13} & g_{14} & g_{15} \\ g_{21} & g_{22} & g_{23} & g_{24} & g_{25} \\ g_{31} & g_{32} & g_{33} & g_{34} & g_{35} \\ g_{41} & g_{42} & g_{43} & g_{44} & g_{45} \\ g_{51} & g_{52} & g_{53} & g_{54} & g_{55} \end{bmatrix} \times \begin{bmatrix} L_{16} & L_{17} & L_{18} \\ L_{26} & L_{27} & L_{28} \\ L_{36} & L_{37} & L_{38} \\ L_{46} & L_{47} & L_{48} \\ L_{56} & L_{57} & L_{58} \end{bmatrix} \tag{4}$$

Clearly, when all the kinetic orders are different from zero, the number of unknowns exceeds the available information, and no unique solution can be derived. In order to be able to estimate a set of parameters, particular subcases of eqn. (4) should be considered, i.e. some of the potential $\mathbf{g}_{ij}$ should be set to zero. However, there are different ways of selecting such a collection of zero kinetic orders, which imply different regulatory structures for the system. To simplify the interpretation, consider the process of synthesis of $X_1(V_1^+)$. Selecting the appropriate elements of eqn. (4) and defining a single parameter vector, we can express:

$$\begin{bmatrix}\mathbf{LV}_{16}\\ \mathbf{LV}_{17}\\ \mathbf{LV}_{18}\end{bmatrix} = \begin{bmatrix}\mathbf{L}_{16} & \mathbf{L}_{26} & \mathbf{L}_{36} & \mathbf{L}_{46} & \mathbf{L}_{56} & 1 & 0 & 0\\ \mathbf{L}_{17} & \mathbf{L}_{27} & \mathbf{L}_{37} & \mathbf{L}_{47} & \mathbf{L}_{57} & 0 & 1 & 0\\ \mathbf{L}_{18} & \mathbf{L}_{28} & \mathbf{L}_{38} & \mathbf{L}_{48} & \mathbf{L}_{58} & 0 & 0 & 1\end{bmatrix} \times \begin{bmatrix}\mathbf{g}_{11}\\ \mathbf{g}_{12}\\ \mathbf{g}_{13}\\ \mathbf{g}_{14}\\ \mathbf{g}_{15}\\ \mathbf{g}_{16}\\ \mathbf{g}_{17}\\ \mathbf{g}_{18}\end{bmatrix} \tag{5}$$

There is no unique solution for the set of kinetic orders in eqn. (5). This is always the case, because $n+m > m$ for any given system. However, in this equation it is assumed that all the variables in the system affect V_1^+, which is unlikely to be true. In general, not all the possible interrelations are meaningful, which implies that some of the potential kinetic orders are zero. Hence, realistic assumptions lead to the consideration of special subcases of eqn. (5). As a general rule, with m independent variables, we can solve for subcases having p variables in V_i, with $p \leqslant m$. Hence, for $n = 5$ and $m = 3$, we can solve for subcases of eqn. (5) with a maximum of three different variables ($p = 3$) affecting a given process. For example:

Case 1: $V_1^+ = \alpha_1 X_1^{g_{11}} X_3^{g_{13}} X_6^{g_{16}}$

$$\begin{bmatrix}\mathbf{LV}_{16}\\ \mathbf{LV}_{17}\\ \mathbf{LV}_{18}\end{bmatrix} = \begin{bmatrix}\mathbf{L}_{16} & \mathbf{L}_{36} & 1\\ \mathbf{L}_{17} & \mathbf{L}_{37} & 0\\ \mathbf{L}_{18} & \mathbf{L}_{38} & 0\end{bmatrix} \times \begin{bmatrix}\mathbf{g}_{11}\\ \mathbf{g}_{13}\\ \mathbf{g}_{16}\end{bmatrix}$$

Case 2: $V_1^+ = \alpha_1 X_4^{g_{14}} X_6^{g_{16}} X_7^{g_{17}}$ (6)

$$\begin{bmatrix}\mathbf{LV}_{16}\\ \mathbf{LV}_{17}\\ \mathbf{LV}_{18}\end{bmatrix} = \begin{bmatrix}\mathbf{L}_{46} & 1 & 0\\ \mathbf{L}_{47} & 0 & 1\\ \mathbf{L}_{48} & 0 & 0\end{bmatrix} \times \begin{bmatrix}\mathbf{g}_{14}\\ \mathbf{g}_{16}\\ \mathbf{g}_{17}\end{bmatrix}$$

Given a measured set of Logarithmic Gains, the two cases considered above lead to two different sets of kinetic orders. Each set corresponds to a different interpretation of the regulatory structure for the considered flux. In each case, we will call the resulting sets of kinetic orders compatible patterns. Hence, in general, there will be no unique compatible solution for the kinetic-order (elasticity coefficients) set. The minimum set of compatible patterns for a given V_i can be obtained by solving for all the possible p subsets ($p \leqslant m$).

However, it should be pointed out that not all the possible p subsets ($p \leqslant m$) are realistic. A necessary condition to obtain a meaningful solution is that Det $[\mathbf{L}_p]$ must be different from zero, $[\mathbf{L}_p]$ being the square matrix of coefficients that multiplies the vector of p unknown kinetic orders in eqn. (6). A determinant equal to zero identifies an impossible combination of parameters given the structure of the Logarithmic-Gain matrices. Application of this rule will permit a first screening of spurious results in investigating the regulatory structure of the system.

The experimental error in the determination of $\mathbf{L}(\mathbf{X_i}, \mathbf{X_k})$ and $\mathbf{L}(\mathbf{V_i}, \mathbf{X_k})$ can influence the estimation of the different kinetic orders in each compatible pattern. This affects the identification of subcases with Det $[\mathbf{L}_p] = 0$. In practice, we will consider values of Det $[\mathbf{L}_p] < 10^{-3}$ to be zero. As we will see in detail in the example, the determination of kinetic orders compatible with a set of Logarithmic Gains is consistent in spite of an experimental error of Logarithmic Gains as large as a $\pm 20\%$ of the true value.

Finally, no unique solution exists if $p > m$. For example, if we consider the simultaneous effect of X_1, X_2, X_3 and X_6 on V_1^+, the corresponding set of equations is:

Case 3: $V_1^+ = \alpha_1 X_1^{g_{11}} X_2^{g_{12}} X_3^{g_{13}} X_6^{g_{16}}$

$$\begin{bmatrix}\mathbf{LV}_{16}\\ \mathbf{LV}_{17}\\ \mathbf{LV}_{18}\end{bmatrix} = \begin{bmatrix}\mathbf{L}_{16} & \mathbf{L}_{26} & \mathbf{L}_{36} & 1\\ \mathbf{L}_{17} & \mathbf{L}_{27} & \mathbf{L}_{37} & 0\\ \mathbf{L}_{18} & \mathbf{L}_{28} & \mathbf{L}_{38} & 0\end{bmatrix} \times \begin{bmatrix}\mathbf{g}_{11}\\ \mathbf{g}_{12}\\ \mathbf{g}_{13}\\ \mathbf{g}_{16}\end{bmatrix} \tag{7}$$

In this case, a solution space can be obtained for three of the kinetic orders involved as a function of the fourth. Hence, complementary information is required to solve these cases. For instance, data from studies *in vitro* could suggest a value for $\mathbf{g}_{16}$. Then, a particular solution corresponding to that assumption can be obtained from eqn. (7). Additionally, we will find other compatible schemes, which are different p subsets ($p = 4$) of eqn. (5), and will contain a particular solution with this value for $\mathbf{g}_{16}$.

In any case, no further discrimination, other than identifying the set of compatible regulatory patterns, is possible by using the steady-state information contained in the Logarithmic-Gains measurements. This emphasizes the limitations of the steady-state data in identifying the regulatory structure without using additional information. Alternative methods based on dynamic data can provide valuable information for discriminating between the different admissible hypotheses in order to identify the true pattern.

Parameter estimation from measurements of transient data obtained in perturbation experiments

S-system parameters can be estimated from experiments in which the initial change in a dependent variable is measured after a given variable has been perturbed from its reference value (Sorribas et al., 1993). In short, if we consider the steady state of the system, we have, for a given X_i ($i = 1, \ldots, n$):

$$\dot{X}_i = 0 \tag{8}$$

Then, after a perturbation in X_k, we can write:

$$\left(\frac{\delta \dot{X}_i}{\delta X_k}\right)_0 \times \frac{X_{k0}}{V_{i0}} = \left(\frac{\delta(V_i^+ - V_i^-)}{\delta X_k}\right)_0 \frac{X_{k0}}{V_{i0}} = g_{ik} - h_{ik} = a_{ik} \tag{9}$$

and:

$$\left(\frac{\delta \dot{X}_i}{\delta X_k}\right)_0 \approx \frac{\Delta \dot{X}_i}{\Delta X_k} = \frac{\dot{X}_{ip} - \dot{X}_{i0}}{X_{kp} - X_{k0}} = \frac{\dot{X}_{ip}}{X_{kp} - X_{k0}} \tag{10}$$

Hence, a_{ik} can be estimated from the steady-state values of the corresponding metabolites and fluxes, and from the initial rate of change of X_i after a perturbation in X_k [see Sorribas et al. (1993) for details]. That is:

$$\hat{a}_{ik} = \frac{\dot{X}_{ip}}{X_{kp} - X_{k0}} \times \frac{X_{k0}}{V_{i0}} \tag{11}$$

This method can be useful from two different points of view. First, we can directly estimate a_{ik} from a perturbation experiment.

Second, we can check the values estimated from the steady-state measurements in order to discriminate between the several possible patterns. This can be achieved by computing the expected $\dot{X}_{ip}$ for a given a_{ik} in eqn. (11) and by comparing the predicted slope with the behaviour observed in a perturbation experiment (see below). This last possibility will allow us to define a suitable strategy to discriminate between the alternative patterns that have been identified from the steady-state characterization.

Strategies for identifying the regulatory structure of a given metabolic pathway

Although the problem of identifying the regulatory pattern of a metabolic pathway can be addressed on the basis of the information accruing from experiments *in vitro*, it is now clear that there is a need for measurements on the intact system that can provide a more reliable image of the structure *in situ*. First, the information *in vitro* may not correspond to the real properties of the integrated system. Second, the information provided by isolated experiments may lead to alternative possibilities that need to be tested on the intact system.

With this in mind, in the previous sections we showed that measurements of the Logarithmic Gain in the intact system do not suffice to identify the regulatory structure underlying the observed behaviour, and that time-course data are required. Hence, a combination of different experimental approaches is needed.

As a general scheme, we suggest the following steps:

1. Draw the scheme accounting for the flow of material through the system. If there are several possibilities, then perform steps 4–6 for each one.
2. Collect information on the possible regulatory signals. Consider both information *in vitro* and hypothetical signals that might be involved in the target system. If possible, compute tentative values for the kinetic orders implicated [for instance using *in vitro* rate laws, or from values of $K_{eq.}$ and mass action ratio (Groen, 1984)].
3. Measure the Logarithmic-Gain characteristics of the system *in situ*, for both fluxes and metabolites.
4. If the system has m independent variables, define the corresponding m subset equations of the general eqn. (3) for all fluxes. Solve for all possible cases and tabulate the compatible patterns.
5. Evaluate each compatible pattern with respect to point 2. Discard all the patterns leading to unrealistic possibilities.
6. Design perturbation experiments leading to the discrimination of alternative patterns. Compute the expected response of the system according to the different alternatives. Perform the perturbation experiments and measure the corresponding dynamic response. Discard the patterns that do not fit the results observed.

Of course, in suggesting this rationale we consider that the appropriate measurements can be performed. In some cases, these measurements may be difficult to complete. In other cases, the number of independent variables available for experimental manipulation may be too limited for the effective identification of the regulatory pattern. However, the possibility of using this approach should encourage the search for appropriate ways of performing the required experiments.

Example

The application of the suggested methodology to a given problem requires measurement of Logarithmic Gains, steady-state levels and transient data. As stated in the Introduction, these measurements, although possible, are not routine in experimental papers, and we know of no single example in which all the required information is available. Hence, we will define a hypothetical system and will discuss the utility of the method with simulated data.

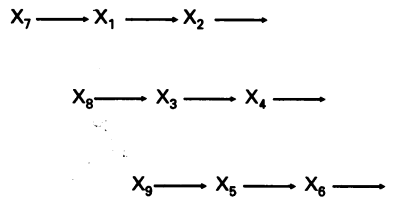

Figure 2. Flow of material through the reference system

X_1 to X_6 are internal metabolites (dependent variables). X_7 to X_9 are external metabolites (independent variables). This system has three independent subsystems that do not interchange material.

As an example, we will consider a hypothetical metabolic pathway with $n = 6$ dependent variables and $m = 3$ independent variables. The scheme for the reference system used and the set of kinetic-order parameters considered are presented in the Appendix. The Logarithmic Gains are computed in the usual way using this hypothetical set of parameters (Sorribas and Savageau, 1989a,b; Cascante et al., 1989a,b). The transient response data in perturbation experiments are obtained by an appropriate numerical procedure using the kinetic equations detailed in the Appendix. Experimental error in measuring the Logarithmic Gains is considered in order to show the robustness of the estimation approach. The influence of this error in estimating a_{ik} in the perturbation experiments is largely discussed in Sorribas et al. (1993).

In the following, we will consider the simulated data as if they had been obtained in a real experiment. These data include a set of Logarithmic Gains for this system and the basic scheme for the flow of material. Furthermore, we consider a set of hypotheses on possible signals in the system. With this information, we will apply the suggested method for identifying the regulatory scheme.

Scheme for the flow of material

The scheme for the flow of material in this example is shown in Figure 2. We can appreciate that, considering the flow of material, we are dealing with three independent pathways.

Available information on possible regulatory signals

The second step is to collect the available information concerning the system. For our example, let us consider this information to be as follows. (1) There is evidence of a positive effect of X_2 on the synthesis of X_3. Besides, X_1 can activate the same reaction *in vitro*. (2) It is suggested that X_6 acts as a feedback inhibitor of the synthesis of X_5. However, no direct evidence has been obtained. (3) There is experimental evidence that an increase in X_4 correlates with an increase in the output flux in the first pathway and a decrease in the third pathway. (4) Although some of the individual enzymes have been isolated, no clear kinetic data are available for computing hypothetic values for the kinetic orders. (5) X_2 is an inhibitor of synthesis of X_1 as has been shown *in vitro*. From available data, a value of $g_{12} = -0.2$ is suggested.

Measurement of the Logarithmic Gains

Our test system produces the following set of Logarithmic Gains:

$$\mathbf{L}(\mathbf{X}_D, \mathbf{X}_I) = \begin{bmatrix} 1.11 & 0.074 & 0.027 \\ 0.73 & -0.34 & -0.13 \\ 0.23 & 1.34 & 0.50 \\ 0.15 & 0.84 & 0.31 \\ -0.11 & -0.66 & 1.11 \\ -0.086 & -0.49 & 0.83 \end{bmatrix} \quad \mathbf{L}(\mathbf{V}_D, \mathbf{X}_I) = \begin{bmatrix} 0.61 & 0.040 & 0.015 \\ 0.61 & 0.040 & 0.015 \\ 0.12 & 0.67 & 0.25 \\ 0.12 & 0.67 & 0.25 \\ -0.075 & -0.43 & 0.72 \\ -0.075 & -0.43 & 0.72 \end{bmatrix} \tag{12}$$

In order to understand these data, recall that the rows indicate a dependent variable (either a metabolite or a flux) and that columns refer to independent variables, i.e. for instance, $\mathbf{L}(\mathbf{X}_2, \mathbf{X}_9) = -0.13$ and $\mathbf{L}(\mathbf{V}_5^+, \mathbf{X}_7) = -0.075$. These values do not take into account experimental error. In order to consider more realistic values, a random noise with Normal distribution was added to the error-free Gains (see below) to test the robustness of the estimation procedure.

From the structure of the data in eqn. (12), it is clear that the three subpathways are interconnected by regulatory signals. This can be concluded because the Logarithmic Gains are all different from zero, indicating a response to changes in independent variables which are not connected by the flow of material through the subsystems.

Compatible patterns

The complete set of compatible patterns with a maximum of three variables for the system considered can be computed by systematically selecting the appropriate subcases of eqn. (3) for each flux. In each case, a different subset of parameter values can be obtained for each subset of parameters considered. Only those subsystems that agree with the flow of material should be considered. This means, for instance, that X_1 must be included in V_1^-, X_7 must be included in V_1^+, and so on. These constraints are evident from the scheme in Figure 2. Additionally, as indicated previously only those subsets leading to a Det $[\mathbf{L}_p]$ different from zero will be considered.

Evaluation of the compatible patterns

Direct inspection of the resulting parameter values shows that some of the potential solutions have no physical meaning. For example, in the process of synthesis of $\mathbf{X}_1$ the subset $[\mathbf{g}_{15}, \mathbf{g}_{16}, \mathbf{g}_{17}]$ yields $\mathbf{g}_{15} = -6899.9$, $\mathbf{g}_{16} = 9235.4$ and $\mathbf{g}_{17} = 1.177$. These values are clearly unrealistic, and should be rejected. Further, in many cases, there is only one significant parameter in a subset of three. This is the case, for instance, of the subcases for V_1^- which reduces to the simpler case $h_{11} = 0.55$.

Besides eliminating these spurious results, the set of compatible subsystems must be checked taking into account the metabolic scheme and the existing evidence on possible signals (see above). Table 1 shows the possible compatible patterns that are physically meaningful for each rate law of synthesis (V_i^+) or degradation (V_i^-) in the exemplary pathway of Figure 2. Further simplification of the alternative patterns will require measurement of the response of the system after a perturbation in an independent variable.

Alternatively, it is possible to consider more than three variables. However, in this case, the resulting equations are not uniquely determined because $m = 3$, and we would have infinite solutions for the parameter set. In such a case, all we can obtain is some of the involved kinetic orders as a function of the others. However, to facilitate the discussion we will not consider this possibility here.

Influence of experimental error

To know how the experimental error on the determination of Logarithmic Gains can affect the evaluation of the compatible patterns, we have performed a simulation study by adding a statistical noise to each of the error-free Logarithmic Gains. The error considered follows a normal distribution with zero mean and σ = true value/10 of the error-free value. This simulates a measurement procedure with an experimental error of $\pm 20\%$ of the true value (95% confidence).

Table 1 Set of compatible regulatory patterns after consideration of the steady-state characterization (see the text)

	Variables			Kinetic orders		
V_1^+	2	7		−0.12	0.70	
	3	7		0.03	0.61	
	4	7		0.05	0.61	
	5	7	8	0.01	0.61	0.05
	5	7	9	−0.06	0.61	0.08
	6	7	8	0.02	0.61	0.05
	6	7	9	−0.08	0.61	0.08
	7	8	9	0.61	0.05	0.02
V_1^-/V_2^+	1			0.55		
V_2^-	2	3		0.77	0.23	
	2	4		0.77	0.36	
	2	5	8	0.86	0.11	0.40
	2	5	9	0.77	−0.46	0.62
	2	6	8	0.86	0.15	0.40
	2	6	9	0.77	−0.61	0.62
	2	8	9	0.84	0.33	0.12
V_3^+	1	5	8	0.13	0.22	0.81
	1	6	8	0.13	0.29	0.81
	1	8	9	0.10	0.66	0.25
	2	5	8	0.20	0.25	0.90
	2	6	8	0.20	0.33	0.90
	2	8	9	0.16	0.72	0.27
	5	7	8	0.22	0.14	0.82
	6	7	8	0.23	0.14	0.82
	7	8	9	0.12	0.67	0.25
V_3^-/V_4^+	3			0.50		
V_4^-	4			0.80		
V_5^+	3	9		−0.32	0.88	
	4	9		−0.51	0.88	
	1	8	9	−0.07	−0.42	0.72
	2	7	9	1.26	−1.00	0.88
	2	8	9	−0.10	−0.46	0.71
	7	8	9	−0.07	−0.43	0.72
V_5^+/V_6^+	5			0.65		
V_6^-	6			0.87		

In Table 2 we show the result of investigating the compatible patterns for V_2^- using 50 simulated experiments. These results show a good agreement between the mean value of these experiments and the error-free value obtained in Table 1. The estimated standard deviation shown in Table 2 also indicates that the estimation of the corresponding kinetic order has a precision comparable with the experimental error of the Logarithmic-Gain determinations. Similar results were obtained for the other fluxes.

To reproduce more closely a real situation, we have simulated experiments with five and three replicates for each Logarithmic-Gain measurement. In Table 3, we show the results for the subset $\{\mathbf{h}_{22}, \mathbf{h}_{24}\}$. In both experiments, the values of the kinetic orders obtained are realistic in spite of the experimental error introduced in the Logarithmic Gains.

Perturbation experiments

In Figures 3–5, we show the transient response of the dependent metabolites after a perturbation in an independent variable. In each case, we estimate the value of a_{ik} using eqn. (11). These data are generated by a numerical procedure using the kinetic equations detailed in the Appendix. In Figure 3(a) we show the computation of the initial slope ($\dot{X}_{ip}$) [see Sorribas et al. (1993) for details]. From the response observed, we can evaluate the competing patterns shown in Table 1.

V_1^+ and V_1^-. No independent variable appears in the patterns of V_1^-. Hence, in this case, a_{ik} reduces to g_{ik}. From Figure 3(a), we have obtained $a_{17} = g_{17} = 0.67$. Additionally, $a_{18} = 0$ and $a_{19} = 0$. Although with this information we cannot rule out cases in which g_{18} and g_{19} are close to zero (Sorribas et al., 1993), it is clear that the value of $g_{17} = 0.67$ is an argument for rejecting the cases in Table 1 having $g_{17} = 0.61$. It was suggested above that X_2 has an inhibitory effect on V_1^+, with a possible value of $g_{12} = -0.2$. This information points towards the case that includes X_2 and X_7 as the only variables to be included in V_1^+.

Table 2 Performance of the estimation procedure

The result of estimating the corresponding parameters in 50 samples obtained by adding a statistical noise with normal distribution of zero main and σ = true value/10 is shown. For each kinetic order the mean (**bold**) values and S.D. (*italics*) of the 50 samples are indicated.

	Variables			Kinetic orders		
V_2^-	2	3		**0.78**	**0.23**	
				0.08	*0.05*	
	2	4		**0.76**	**0.36**	
				0.11	*0.05*	
	2	5	8	**0.85**	**0.12**	**0.43**
				0.12	*0.03*	*0.09*
	2	5	9	**0.78**	**−0.48**	**0.66**
				0.10	*0.08*	*0.14*
	2	6	8	**0.84**	**0.15**	**0.41**
				0.12	*0.03*	*0.08*
	2	6	9	**0.77**	**−0.64**	**0.67**
				0.10	*0.11*	*0.14*
	2	8	9	**0.86**	**0.34**	**0.13**
				0.14	*0.07*	*0.03*

Table 3 Performance of the estimation procedure in small samples

The results are obtained with the same procedure as in Table 2. For each kinetic order the mean (**bold**) value and S.D. (*italics*) are indicated.

	Variables		Number of samples	Kinetic orders	
V_2^-	2	4	5	**0.79**	**0.36**
				0.11	*0.07*
			3	**0.72**	**0.33**
				0.08	*0.05*

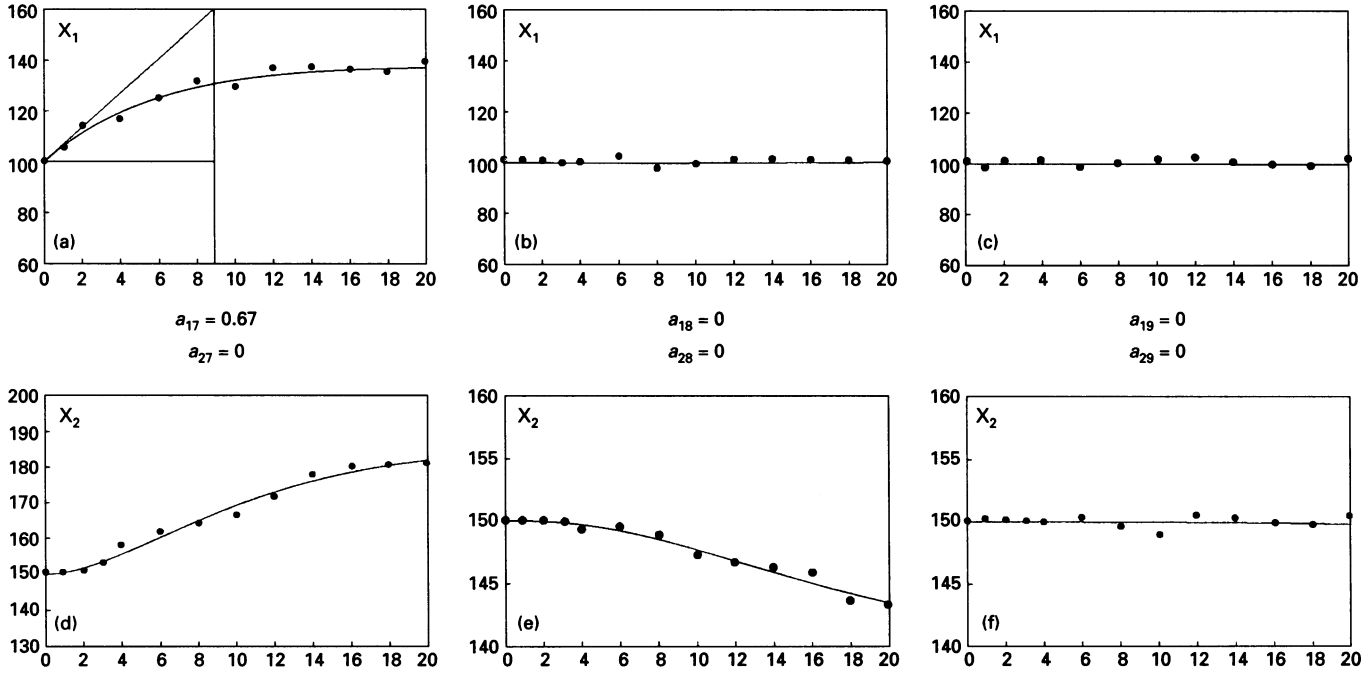

Figure 3. Response of X_1 and X_2 after a perturbation in an independent variable

(**a, d**) X_7 changes from 300 to 400; (**b, e**) X_8 changes from 500 to 600; (**c, f**) X_9 changes from 400 to 500.

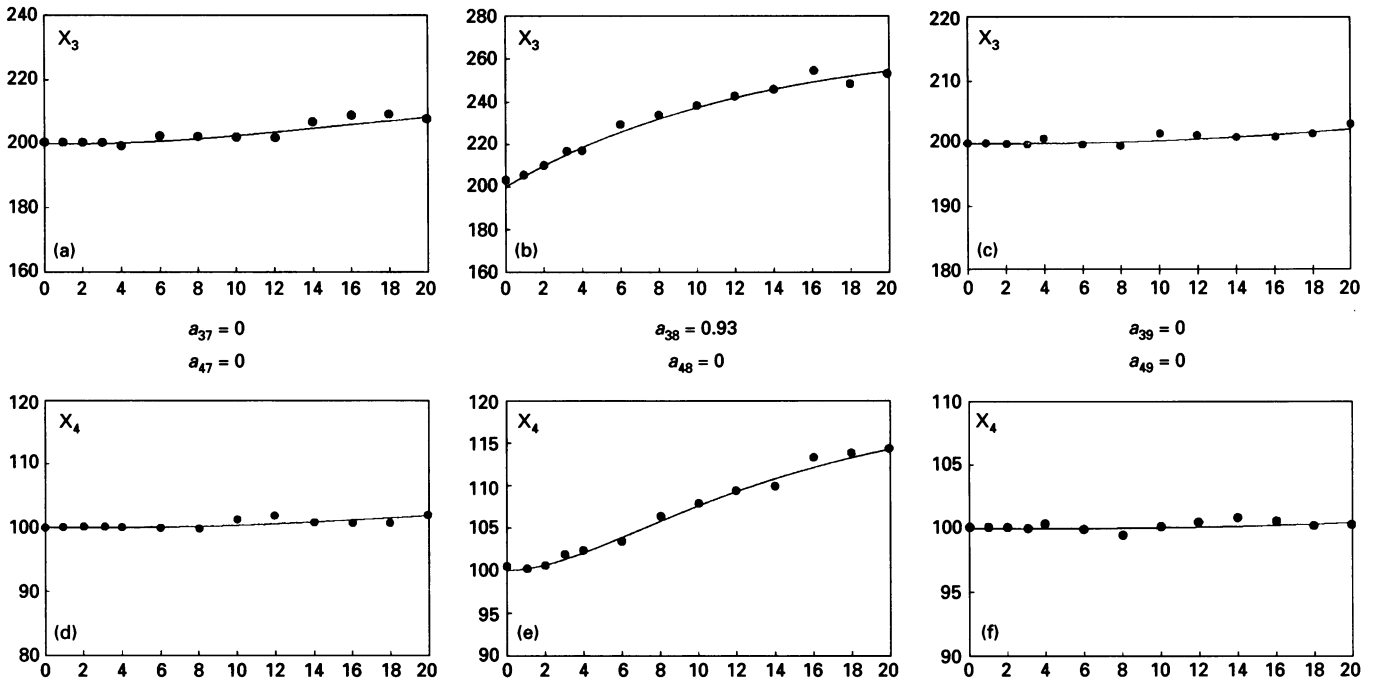

Figure 4. Response of X_3 and X_4 after a perturbation in an independent variable

(**a**, **d**) X_7 changes from 300 to 400; (**b**, **e**) X_8 changes from 500 to 600; (**c**, **f**) X_9 changes from 400 to 500.

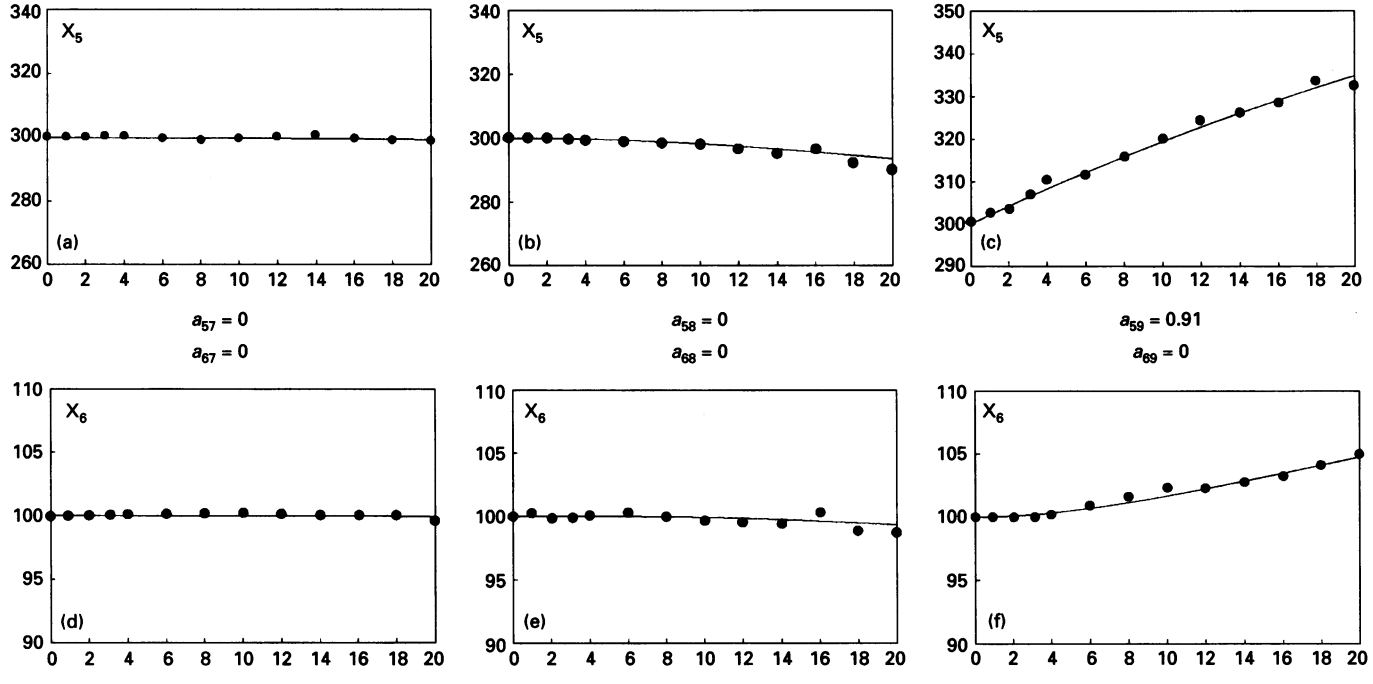

Figure 5. Response of X_5 and X_6 after a perturbation in an independent variable

(**a**, **d**) X_7 changes from 300 to 400; (**b**, **e**) X_8 changes from 500 to 600; (**c**, **f**) X_9 changes from 400 to 500.

V_2^+ and V_2^-. Because no independent variable has been selected in the compatible patterns for V_2^+, a_{2k} reduces to $-h_{2k}$. In V_2^- only X_8 and X_9 appear in the patterns of Table 1. From the results obtained in Figures 3(d)–3(f), it is clear that neither X_8 nor X_9 has a direct effect on V_2^-. Hence, the two cases shown in Table 4 result.

V_3^+ and V_3^-. From Figure 4(a), we have obtained $a_{37} = 0$. From the subcases in Table 1, the possibilities that include X_7 lead to $g_{37} - h_{37} = 0.14 - 0.24 = -0.1$, or $g_{37} - h_{37} = 0.12 - 0.24 = -0.12$, which do not correspond to the behaviour observed in Figure 4(a). With this argument, and with the values of a_{38} and a_{39}, the patterns shown in Table 4 are selected.

V_4^+ and V_4^-. From the above discussion, and considering that $V_3^- = V_4^-$, the results of Figure 3(d)–3(f) lead to a single possibility

Table 4 Final set of compatible patterns of regulation after the perturbation experiments (see the text)

	Variables			Kinetic orders		
V_1^+	2	7		−0.12	0.70	
V_1^-/V_2^+	1			0.55		
V_2^-	2	3		0.77	0.23	
	2	4		0.77	0.36	
V_3^+	2	5	8	0.20	0.25	0.90
	2	6	8	0.20	0.33	0.90
V_3^-/V_4^+	3			0.50		
V_4^-	4			0.80		
V_5^+	3	9		−0.32	0.88	
	4	9		−0.51	0.88	
V_5^-/V_6^+	5			0.65		
V_6^-	6			0.87		

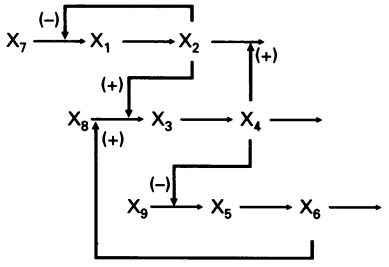

Figure 6. Reference system

X_7, X_8 and X_9 are independent variables. Arrows with a (+) indicate an activatory effect. Arrows with (−) indicate an inhibitory effect.

for the degradation of X_4, in which the only variable is X_4 itself.

V_5^+ and V_5^-. Because $a_{57} = 0$ and $a_{58} = 0$, the only possibilities after the results shown in Figures 5(a)–5(c) are those indicated in Table 4.

V_6^+ and V_6^-. There is no independent variable included in the pattern selected for these reactions in Table 4. The results shown in Figures 5(d)–5(f) confirm this.

After these considerations, Table 4 summarizes the compatible situations that match the perturbation experiments. As shown in this Table, there are still a few alternatives after these experiments. First, in the synthesis of X_2, two alternatives appear having X_3 or X_4 as positive effectors. Because there is some evidence of a correlation of an increase in this rate after an increase in X_4 (see above), we can tentatively consider that X_4 is the variable involved. Second, we have a similar situation with the synthesis of X_5. In this case, we can also decide that X_4 is the variable involved, following the information considered above. Alternatively, and if X_4 can be perturbed from its steady state, the values of h_{24} and g_{54} can be measured experimentally to test these assumptions. Finally, there is no information on which to decide between the alternative patterns for the synthesis of X_3 in Table 4. At this point, only direct measurement of g_{35} or g_{36} can lead to a decision. Alternatively, we can consider isolating this enzyme and testing which metabolite acts as an activator of this reaction *in vitro*. The results shown in Table 4, and the final considerations stated above, should be compared with the test system (Figure 6) and the real parameter values indicated in the Appendix.

DISCUSSION

Investigation of the properties of a given metabolic pathway requires definition of an appropriate strategy of data analysis so that the observed behaviour can be related to an appropriate description of the system structure. We have shown that the information contained in the steady-state measurements can be processed to produce a set of tentative interpretations of the regulatory structure of the system. However, it is clear that this information is not enough to yield a unique solution. In fact, for a given set of Logarithmic-Gain measurements, we have shown that different sets of regulatory signals can explain the behaviour observed. This multiplicity of compatible regulatory patterns makes it necessary to consider a way of identifying the true pattern.

The method introduced in Sorribas et al. (1993) can help to solve this problem by focusing on the measurement of the initial rate of change in a given dependent variable after a perturbation in any of the variables considered in the problem. This method is particularly indicated for discrimination between the different alternative patterns compatible with the steady-state behaviour. In this sense, the steady-state approach helps in designing the appropriate perturbation experiments, so that the experimental effort required to identify the regulatory pattern can be dramatically reduced.

The results presented in this paper show that the actual regulatory pattern can be identified by following a step-by-step procedure. In principle, the suggested strategy can be applied to any system, provided that the required measurements are available. In the presence of experimental error, we have shown that a consistent estimation can be obtained, so that an approximate description of the system can be derived. We are aware of the difficulties of performing some of the required measurements in specific cases. By showing the possibility of identifying the regulatory structure, which is a legitimate goal in metabolic research, this paper should encourage experimentalists to develop new techniques for obtaining the required data. In this sense, theoretical studies can open up new ways of addressing key questions. The suggestion of specific measurements optimizing the search for a regulatory structure contributes a new way of looking at this kind of problem.

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APPENDIX

Reference system

The reference system used to generate the data considered in the Example section is shown in Figure 6 of the main paper. The aim is not to represent a particular metabolic situation but to provide a suitable example to validate the recommended methodology. To simulate experimental data, this system is modelled by using irreversible Michaelis rate laws. The kinetic parameters considered for each reaction are the following:

$X_1 \rightarrow X_2 \quad K_m = 122.2 \quad V_{max.} = 11.1$
$X_3 \rightarrow X_4 \quad K_m = 200 \quad V_{max.} = 60$
$X_4 \rightarrow \quad K_m = 400 \quad V_{max.} = 150$
$X_5 \rightarrow X_6 \quad K_m = 557.1 \quad V_{max.} = 28.6$
$X_6 \rightarrow \quad K_m = 669.2 \quad V_{max.} = 76.9$

The inhibition of X_2 (or X_4) on the synthesis of X_1 (or X_5) is represented by the following rate law:

$$V_h^+ = \frac{V_{max.h} X_j}{K_{m_h}\left(1+\frac{X_l}{K_{i_l}}\right)+X_j}$$

where, for the synthesis of X_1, $h = 1$, $j = 7$, $l = 2$, $K_{m_1} = 580$, $V_{max.1} = 100$ and $K_{i_2} = 725$; and, for the synthesis of X_5, $h = 5$, $j = 9$, $l = 4$, $K_{m_5} = 1233.3$, $V_{max.5} = 83.3$ and $K_{i_4} = 72.6$.

The activation of X_4 on the degradation of X_2 is represented by the following rate law:

$$V_2^- = \frac{V_{max.2} X_2\left(1+\frac{\beta_4 X_4}{K_{a_4}}\right)}{(K_{m_2}+X_2)\left(1+\frac{X_4}{K_{a_4}}\right)}$$

where $K_{m_2} = 502.2$, $V_{max.2} = 21.3$, $K_{a_4} = 75.5$ and $\beta = 10$.

The activation of X_2 and X_6 on the synthesis of X_3 is represented by the following rate law:

$$V_3^+ = \frac{V_{max.3} X_8\left(1+\frac{\beta_2 X_2}{K_{a_2}}+\frac{\beta_6 X_6}{K_{a_6}}\right)}{(K_{m_3}+X_8)\left(1+\frac{X_2}{K_{a_2}}+\frac{X_6}{K_{a_6}}\right)}$$

where $K_{m_3} = 4500$, $V_{max.3} = 66.9$, $K_{a_2} = 848.9$, $K_{a_6} = 349.0$, $\beta_2 = 12$ and $\beta_6 = 12$.

Data used in the perturbation experiments are generated by a numerical procedure using the above kinetic equations.

The S-system equations for this system, after considering that $V_1^- = V_2^+$, $V_3^- = V_4^+$ and $V_5^- = V_6^+$, are:

$$\dot{X}_1 = \alpha_1 X_2^{g_{12}} X_7^{g_{17}} - \beta_1 X_1^{h_{11}}$$
$$\dot{X}_2 = \beta_1 X_1^{h_{11}} - \beta_2 X_2^{h_{22}} X_4^{h_{24}}$$
$$\dot{X}_3 = \alpha_3 X_2^{g_{32}} X_6^{g_{36}} X_8^{g_{38}} - \beta_3 X_3^{h_{33}}$$
$$\dot{X}_4 = \beta_3 X_3^{h_{33}} - \beta_4 X_4^{h_{44}}$$
$$\dot{X}_5 = \alpha_5 X_4^{g_{54}} X_9^{g_{59}} - \beta_5 X_5^{h_{55}}$$
$$\dot{X}_6 = \beta_5 X_5^{h_{55}} - \beta_6 X_6^{h_{66}}$$

Under the conditions considered, the steady-state values for the dependent metabolites $X_1 - X_6$ are 100, 150, 200, 100, 300 and 100 (arbitrary units). The independent variables X_7, X_8 and X_9 are equal to 300, 500 and 400 in basal conditions. The steady-state flux is equal to 30 in the first and second subsystems, and equal to 10 in the third. With these conditions, the set of S-system parameters are:

$$\dot{X}_1 = 1.01 X_2^{-0.12} X_7^{0.70} - 2.38 X_1^{0.55}$$
$$\dot{X}_2 = 2.38 X_1^{0.55} - 0.12 X_2^{0.77} X_4^{0.36}$$
$$\dot{X}_3 = 0.009 X_2^{0.20} X_6^{0.33} X_8^{0.90} - 2.12 X_3^{0.50}$$
$$\dot{X}_4 = 2.12 X_3^{0.50} - 0.75 X_4^{0.80}$$
$$\dot{X}_5 = 0.54 X_4^{-0.51} X_9^{0.88} - 0.24 X_5^{0.65}$$
$$\dot{X}_6 = 0.24 X_5^{0.65} - 0.18 X_6^{0.87}$$

These parameters are the target parameters to be estimated by using experimental data. The resulting values shown in Tables 1–4 should be compared with these reference values.

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