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Metabolic Pathway Characterization from Transient Response Data Obtained *In Situ*: Parameter Estimation in S-system Models

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The actual values of internal metabolites and fluxes can be measured by a number of experimental techniques and they provide important information for evaluating the properties of a metabolic pathway *in situ*. In this paper we propose a strategy to properly exploit this information. The suggested approach permits estimation of a set of parameters on the whole system so that a useful model can be constructed and used to describe its components and systemic properties and to predict its behavior under new conditions. A simulated reference pathway is provided to validate this method and to show its utility in metabolic studies.

Introduction

Experimental determination of levels of metabolites *in situ* and their rate of variation with time is now available by a number of techniques. In experiments with permeabilized cells, the response to variations in external conditions or to perturbations in internal metabolites can be measured without significant modifications of the *in vivo* conditions (see examples in Jorgeson & Nordlie, 1980; Choudary, 1984; Gowda *et al.*, 1988; and references in the review by Felix, 1982). A second class of experiments involves NMR spectroscopy, which allows for direct determination of metabolites *in vivo* (den Hollander & Shulman, 1983; Shulman, 1983, 1988; Cerdan & Seeling 1990; Jeffrey *et al.*, 1991). This technique allows for a simultaneous recording of different metabolites by using a single spectrum or by combining different alternative spectra based on ^{3}H,^{31}P,^{13}C,^{23}Na,^{39}K and other isotopes (Cohen, 1983; Campbell-Burk & Shulman, 1987; Campbell-Burk *et al.*, 1987; O'Fallon & Wright 1987; Kuchel *et al.*, 1990). Because of these properties, there are an increasing number of metabolic questions that have been addressed by this technique either qualitatively or by using mathematical models (Cohen, 1987*a*, *b*; Hutson *et al.*, 1988; Laughlin, 1988; Malloy *et al.*, 1990; Jans & Willem, 1991; Sugden & Fuller, 1991; see also Cerdan & Seeling, 1990; Kuchel *et al.*, 1990; Jeffrey *et al.*, 1991 for recent applications). All these experimental approaches can be used to record the time course of metabolite changes

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after a particular perturbation of the operating steady state (see Katz *et al.*, 1979; den Hollander *et al.*, 1979, 1981, 1986; Sillerud & Shulman, 1983; Galazzo & Bailey, 1989; Houwen *et al.*, 1991).

The kinetic characterization of an isolate enzyme from dynamic data is possible even when more than one substrate or inhibitor is involved. The methodology for achieving such characterization is well defined and can be found in the literature (Cornish-Bowden, 1976; Canela & Franco, 1986). In contrast, when data collected in the whole system *in situ* are used, the information contained in the recorded time course of the metabolite changes after a particular perturbation is not interpreted as easily as in isolated experiments. In the intact system, changes in metabolite levels should not be fitted to an individual rate law equation. These changes are a consequence of the balance of the different rate laws of synthesis and degradation of the involved metabolites and concerns different enzyme reactions. In consequence, a different strategy and an adequate systemic approach are needed to use this information in characterizing the appropriate set of parameters so that a mathematical model allowing for a complete characterization of the system in the studied conditions can be defined.

An appropriate tool that permits construction of a workable model of the system behavior (i.e. incorporation of all the relevant interactions of the system, adequate representation of the component properties and of the system as a whole, and suitable analysis of the properties of the system) is based on the concepts of the *power-law* formalism and has led to mathematical models in the form of S-systems (Savageau, 1969, 1972, 1974, 1975, 1976; Voit & Savageau, 1982, 1987; Cascante *et al.*, 1991; see also Savageau *et al.*, 1987*a*, *b*; Cascante *et al.*, 1989*a*, *b*; Savageau & Sorribas, 1989; Sorribas & Savageau, 1989*a*, *c*; for how to relate this approach to other techniques based on sensitivity coefficients). The S-system methodology provides a systematic way of building a mathematical representation of a biochemical pathway by focusing in its systemic properties. In this approach, individual reactions are aggregated into net processes accounting for the synthesis and degradation of each internal metabolite (Savageau, 1969, 1976; Voit & Savageau, 1982, 1987; Sorribas & Savageau, 1989*a*, *c*). After aggregation, a power-law representation of each aggregated process gives the S-system representation (see Savageau, 1976; Voit & Savageau, 1982, 1987; Sorribas & Savageau, 1989*a*, *c*, for discussion of the optimal strategies for building this representation).

The S-system equations allow for a complete steady-state characterization of the system by means of *logarithmic gains* (for example, response of an internal metabolite to changes in an independent metabolite) and *parameter sensitivities* (for example, response of an internal metabolite to change in a system parameter) (Savageau, 1971 *a*, *b*, 1972,1974,1975,1976; Savageau & Sorribas, 1989; Sorribas & Savageau, 1989*a*, *b*, *c*). It also allows analysis of the dynamic response and the comparison of alternative pathway designs which results in predictions about their optimal organization based on defined criteria for functional effectiveness (Savageau, 1972, 1974, 1975, 1979, 1985; Irvine & Savageau, 1985*a*, *b*).

The S-system models are characterized by a set of parameters that include generalized kinetic orders and rate constants. Their relationship to the usual enzyme kinetic parameters have been discussed elsewhere (Savageau, 1976; Voit & Savageau,

1987; Savageau, 1991*b*). In that sense, it is important to stress that the S-system models are not an alternative to mechanistic rate laws for studying enzyme mechanisms. S-system models are an alternative to study the system as a whole and they use a novel point of view on the representation of the system components (Savageau, 1991*b*). Following this outlook, several strategies based on steady-state measurements have been devised for estimating S-system parameters (Savageau, 1976; Savageau *et al.*, 1987*b*; Voit *et al.*, 1991). Particularly, the computation of the kinetic orders has been addressed by a number of experimental procedures that undertake experimental modification of the system (Kacser & Burns, 1979; Groen *et al.*, 1982, 1986; Wanders *et al.*, 1983; Groen, 1984; Torres *et al.*, 1986, 1988; Canela *et al.*, 1990; Torres & Meléndez-Hevia, 1991)†. Several solutions have also been suggested for the estimation problem using dynamic data (Voit & Savageau, 1982; Johnson, 1988, 1991; Torsella & Bin Razali, 1991). For these methods, rather accurate measurements and initial guesses of the parameter values are required to obtain good estimates (Voit & Savageau, 1982; Torsella & Bin Razali, 1991). In many experimental situations, however, measurements are restricted to initial changes, which results in ill-conditioned data that limits the application of the preceding methods (Torsella & Bin Razali, 1991).

In this paper we propose a new strategy to estimate the kinetic order parameters from experimental measurements of the initial rates of change in the intact system. To assess the performance of the methodology suggested, we shall compare the behavior and properties of a reference system with the predictions made by the S-system equations with the estimated parameter set. In addition, we will briefly review how to construct a model based on the S-system equations and how it can be used to account for the component and system properties and for predicting the system behavior under new conditions.

Methods

REFERENCE SYSTEM AND SIMULATED EXPERIMENTAL DATA

As a reference system we shall use the metabolic pathway shown in Fig. 1. This is not aimed at representing a particular metabolic situation but to provide a suitable example to validate the recommended methodology. To simulate experimental data, this system is modeled by using irreversible Michaelis rate-laws. The feedback inhibition of X_4 on the degradation of X_1 and X_2 is represented by the following rate law:

$$v_{3i}=\frac{v_{3i}X_i}{K_{m_i}\left(1+\frac{X_4}{K_{i4}}\right)+X_i\left(1+\frac{X_4}{K'_{i4}}\right)}, \qquad i=1,2 \tag{1}$$

† These methods were devised within the Metabolic Control Theory methodology, which is closely related with the S-system approach based in the power-law formalism (see Savageau *et al.*, 1987*a*, *b*; Sorribas & Savageau, 1989*a*, *b*; Savageau, 1991*a* for discussion). Hence, they can be considered as estimation methods for the kinetic orders, since there is a clear equivalence with the elasticity coefficients defined in Metabolic Control Theory.

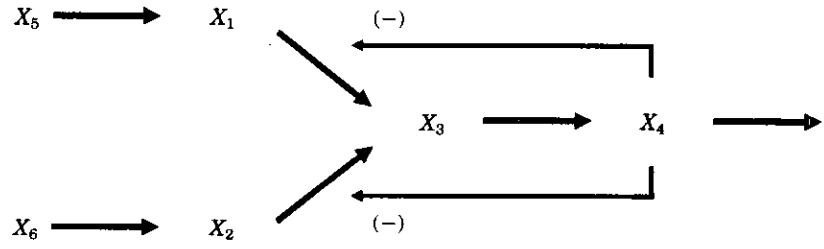

FIG. 1. Reference system. X_1 to X_4 are dependent metabolites. X_5 and X_6 are source metabolites (independent variables). Dotted lines indicate feedback inhibition on the processes of synthesis of X_3, exerted by X_4. Metabolic levels and fluxes at the operating steady state considered are indicated in Table 2.

where v_{3i} indicates the rate of synthesis of X_3 from X_i. The kinetic parameters considered and the resulting steady-state values of metabolite concentrations and fluxes are indicated in Table 1. We have considered the particular case in which $K'_{i4} \gg X_4$.

Data used in the estimation routine are generated by a numerical procedure using the kinetic equations [eqn (1)]. Data with experimental measurement errors are generated by adding a statistical noise with normal distribution, zero mean and a standard deviation equal to 2·5% of the error-free computed value. This simulates a measurement procedure with an experimental error of ±5% of the true concentration value (95% confidence), which is in agreement with the typical experimental error reported in using NMR techniques.

TABLE 1

Kinetic parameters and steady-state values for the reference system in Fig. 1

Reaction	V	K_m	K_{i4}
$X_5 \rightarrow X_1$	3	100	—
$X_6 \rightarrow X_2$	3	250	—
$X_3 \rightarrow X_4$	3	1	—
$X_4 \rightarrow$	3	2	—
$X_1 \rightarrow X_3$	5·33	6·67	6·67
$X_2 \rightarrow X_3$	5·50	15	2

Variable	Steady state
X_1	2
X_2	3
X_3	1
X_4	2
X_5	50
X_6	50
$V_{+1} = V_{-1}$	1
$V_{+2} = V_{-2}$	0·5
$V_{+3} = V_{-3}$	1·5
$V_{+4} = V_{-4}$	

S-SYSTEM EQUATIONS

The S-system representation is used as the basis for characterizing the reference system. Following the well-established procedure for building up this representation (see for instance, Savageau, 1969, 1976; Voit & Savageau, 1982; and Sorribas & Savageau, 1989*a*, *c*, for a detailed discussion on the rationale for writing these equations), for a metabolic pathway with n dependent variables (metabolites, enzymatic forms, etc) and m independent variables (pathway substrates and products, enzymes that do not vary significantly with the behavior of the system, external effectors, etc), the S-system equations are:

$$\frac{dX_i}{dt} = \dot{X}_i = V_i - V_{-i} = \alpha_i \prod_{j=1}^{n+m} X_j^{g_{ij}} - \beta_i \prod_{j=1}^{n+m} X_j^{h_{ij}}, \qquad i = 1,.., n. \tag{2}$$

In this representation the parameters are g_{ij}, h_{ij} (*kinetic orders*), α_i and β_i (*rate constants*). The kinetic orders are the target parameters which must be estimated from experimental measurements. These parameters correspond to the relative change of $V_i(g_{ij})$ or $V_{-i}(h_{ij})$ as a result of a change in X_j when the other variables are kept constant at their operating values. Once kinetic orders are obtained, rate constants can be computed from the kinetic orders and from the operating values of fluxes and metabolites (see Savageau, 1976 or Sorribas & Savageau, 1989*a*, and references therein, for a detailed account of the meaning of these parameters).

STEADY-STATE CHARACTERIZATION

Following the usual methodology in analyzing S-system models, the operating steady-state is characterized by means of *logarithmic gains* and *parameter sensitivities*. Logarithmic gains are defined as the logarithmic derivatives of the dependent variables with respect to an independent variable and they measure the percentage response in the steady-state level of a dependent variable after a change in an independent variable. Parameter sensitivities are defined as the logarithmic derivatives of the dependent variables with respect to a parameter and they measure the response to a change in a parameter of the system (rate constant or kinetic order). Both logarithmic gains and parameter sensitivities measure a *systemic* response, that is a property of the system as a whole (see Savageau, 1976; Irvine & Savageau, 1985*a*, *b*; Sorribas & Savageau, 1989*a*, *c* for examples). To compute the logarithmic gains and the parameter sensitivities we need to know the steady-state values of the metabolites and fluxes considered, and the values of the kinetic orders and rate constants (Savageau, 1971*a*, *b*, 1972, 1976; Savageau *et al.*, 1987*a*, *b*; Savageau & Sorribas, 1989; Sorribas & Savageau, 1989*a*, *c*). In practice, once these values are known the steady-state characterization and the dynamic simulations can easily be obtained by using the program ESSYNS, which was especially devised to analyze the S-system equations‡ (Voit *et al.*, 1989; Irvine & Savageau, 1990).

‡ This program is available upon request to: E. O. Voit, Department of Biostatistics, Epidemiology and System Science, Medical University of South Carolina, Charleston, SC 29425–2503, U.S.A.

STATISTICAL PROCEDURES

The REGRESSION procedure in the statistical program SPSS/PC$^+$ v.4.0 was used to fit the polynomials in estimating the initial rates of change in each of the simulated conditions studied. Statistical noise, when needed, was generated by using the SPSS/PC$^+$ built in algorithms.

Results

ESTIMATING S-SYSTEM PARAMETERS FROM DYNAMIC DATA

In this section, we develop a procedure for estimating the S-system parameters from the initial change in metabolite concentration after a perturbation is produced experimentally. First, we show how S-system parameters are related with the dynamic response after a perturbation. Second, we set up a procedure for computing these parameters from experimental data.

Relating dynamic responses to S-system parameters

In a particular steady state, indicated by the subscript $_0$, the time derivatives are equal to zero, that is, for each dependent metabolite X_i, the rate of synthesis (V_{i0}) is equal to the rate of degradation (V_{i0}):

$$\dot{X}_{i0} = V_{i0} - V_{-i0} = 0, \qquad i = 1,.., n. \tag{3}$$

After a perturbation is introduced in any of the variables of the system, say X_k, a change in the steady-state values will be observed. For any dependent variable X_i in which X_k appears as a variable affecting its synthesis or its degradation (that is, either g_{ik} or h_{ik} is different from zero), the change in the net flux through X_i evaluated at the operating point can be written as:

$$\left(\frac{\partial \dot{X}_i}{\partial X_k}\right)_0 \cdot \frac{X_{k0}}{V_{i0}} = \left(\frac{\partial (V_i - V_{-i})}{\partial X_k}\right)_0 \cdot \frac{X_{k0}}{V_{i0}} = g_{ik} - h_{ik} = a_{ik}. \tag{4}$$

Hence, the differences between kinetic orders ($g_{ik} - h_{ik}$) can be evaluated if a suitable measurement of the change in the time derivative is provided. If we consider a small perturbation in X_k, we can write:

$$\left(\frac{\partial \dot{X}_i}{\partial X_k}\right)_0 \approx \frac{\Delta \dot{X}_i}{\Delta X_k} = \frac{\dot{X}_{ip} - \dot{X}_{i0}}{X_{kp} - X_{k0}} \tag{5}$$

where the subindex $_p$ refers to the perturbed values. If we take into account eqn (3), then eqn (5) reduces to:

$$\left(\frac{\partial \dot{X}_i}{\partial X_k}\right)_0 = \frac{\dot{X}_{ip}}{X_{kp} - X_{k0}}. \tag{6}$$

According to this result, we must evaluate the initial rate of change in X_i after a perturbation in X_k in order to estimate a_{ik}.

Evaluation of the initial rate of change of a dependent metabolite

The slope of the time course of X_i after a perturbation of X_k, evaluated at the time in which we perturb the system ($t=0$), will give us the initial rate of change in X_i. The graphical meaning of the initial slope is shown in Fig. 2. To evaluate this quantity in experimental data a statistical procedure is needed. A second order polynomial

TABLE 2

Estimation of the initial rate of change (c_1) *in the concentration of each dependent metabolite* [*cf. eqn* (7)]

Perturbed variable	%	Observed variable	Estimated initial rate (c_1)
X_1	100	X_1	−0·685
	25		−0·193
	15		−0·118
X_1	100	X_3	0·663
	25		0·188
	15		0·115
X_2	100	X_2	−0·419
	25		−0·112
	15		−0·068
X_2	100	X_3	0·407
	25		0·108
	15		0·065
X_3	100	X_3	−0·508
	25		−0·169
	15		−0·106
X_3	100	X_4	0·499
	25		0·165
	15		0·103
X_4	100	X_1	0·159
	25		0·046
	15		0·028
X_4	100	X_2	0·157
	25		0·051
	15		0·032
X_4	100	X_3	−0·309
	25		−0·094
	15		−0·058
X_4	100	X_4	−0·509
	25		−0·171
	15		−0·107
X_5	100	X_1	0·499
	25		0·154
	15		0·096
X_6	100	X_2	0·357
	25		0·100
	15		0·061

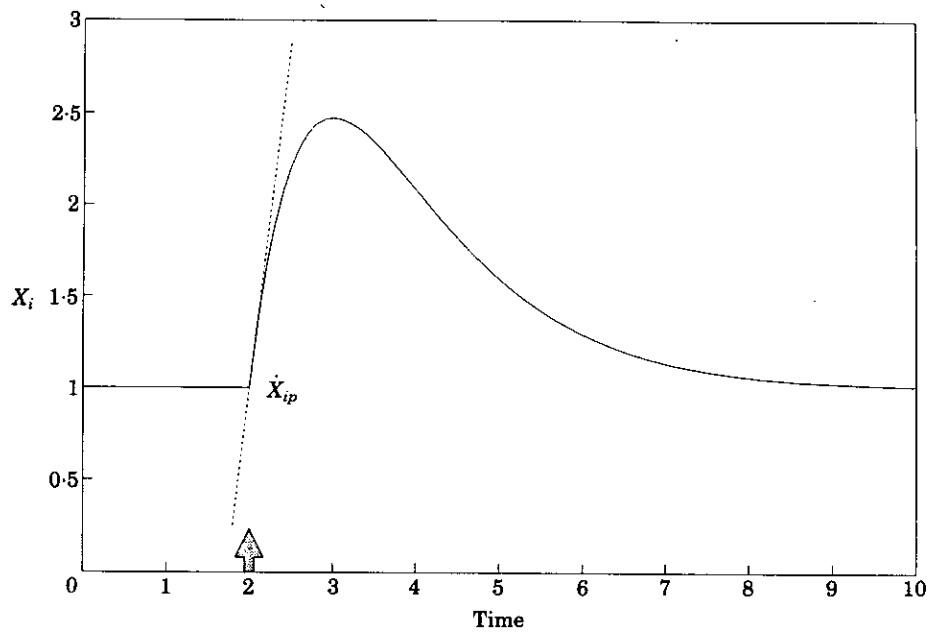

FIG. 2. Initial rate of change on X_i after a perturbation in X_k. From the dynamic response of X_i after a perturbation in X_k we can obtain the value of the initial rate of change by measuring the slope of this dynamic response at the time of perturbation.

regression leads to results that are accurate enough for practical purposes§. For a set of data collected at different times after the perturbation, the observed response can be aproximated by:

$$\dot{X}_i(t) = X_{i0} + c_1 \,.\, t + c_2 \,.\, t^2. \tag{7}$$

Once eqn (7) has been fitted to the observed data set, the initial rate of change at $t=0$, can be obtained as:

$$\dot{X}_{ip} = \left(\frac{\mathrm{d}X_i(t)}{\mathrm{d}t}\right)_0 = \hat{c}_1 \,. \tag{8}$$

To obtain a good estimation of this slope, it is not necessary to follow the dynamics over a large interval of time. This interval can be quite narrow if we can measure metabolite levels at time points close enough to the perturbation time.

§ We use second order polynomial regression because it is appropriate according to the shape of the observed response at times close to $t=0$. A higher order polynomial did not add accuracy to the estimated parameters. In some cases, and specially when the experimental data are collected over a large time range, the use of a third order polynomial could be necessary. Alternatively, the initial slope can be obtained by other techniques such as non-parametrical procedures, especially if the polynomial fit does not mimic the observed response.

Computing the S-system parameters

According to eqns (4), (6) and (8), an estimation of the difference $(g_{ik}-h_{ik})$ can be obtained as:

$$\hat{a}_{ik}=(\hat{g}_{ik}-\hat{h}_{ik})=\frac{\hat{c}_i}{X_{kp}-X_{k0}}\cdot\frac{X_{k0}}{V_{i0}}. \tag{9}$$

Individual values for g_{ik} and h_{ik} can be obtained from a_{ik} if X_k affects only the synthesis ($h_{ik}=0$) or the degradation ($g_{ik}=0$) of X_i. In the case in which X_k affects both processes, it is necessary to look at the precursor–product relationships in order to identify an appropriate set of a_{ik} so that the individual kinetic orders could be computed.

Application: A Simulation Study on the Performance of the Estimation Procedure

In the preceding section, we have developed a method for estimating the S-system parameters from experimental data. In order to validate the suggested methodology, and to show the potential utility of this approach, we investigate a reference system with simulated experiments. The reference system and the simulation procedure have been defined in the Methods section (Fig. 1). After showing the performance of the method, we shall discuss some of the properties of this system that can be elucidated with the S-system methodology.

S-system representation of the reference system

The S-system representation of the reference system can be directly derived from the scheme in Fig. 1. Rules for setting up the equations have been presented several times (see, for example, Savageau, 1969, 1976; Voit & Savageau, 1982; Sorribas & Savageau, 1989*a*, *c*). However, we will briefly indicate how to proceed in our reference system so that the forthcoming sections can be understood properly. In this example, the process of synthesis of X_1 is the reaction producing this metabolite from X_5. As appears in the scheme, the only metabolite affecting this reaction is X_5. Hence, the representation of this process includes a rate constant (α_1) and a term in X_5 raised to g_{15} [see eqn (12)]. This parameter corresponds to the relative response of this process to a change in X_5. Similarly, the degradation of X_1 depends both on X_1 (the substrate of the reaction) and X_4 (an inhibitor). Hence, the representation of this process includes a rate constant (β_1) and two terms (one for each variable affecting the considered process): one for X_1 raised to h_{11} and another for X_4 raised to h_{14}. The kinetic orders h_{11} and h_{14} are parameters that relate the response of this process to a change in the variables considered. To build the other equations we follow the same technique. We note that the synthesis of X_3 results from two different processes: one producing X_3 from X_1 and another producing X_3 from X_2. In this case, we define an aggregate rate of synthesis of X_3 that is affected by X_1, X_2 and X_4. The representation of this process includes a rate constant (α_3) and three terms, one for each of the variables involved, raised to their corresponding kinetic orders (g_{31},

g_{32} and g_{34}). The aggregation procedure results in the following product–precursor relationship:

$$\alpha_3 X_1^{g_{31}} X_2^{g_{32}} X_4^{g_{34}} = \beta_1 X_1^{h_{11}} X_4^{h_{14}} + \beta_2 X_2^{h_{22}} X_4^{h_{24}}. \tag{10}$$

The parameters involved are not independent because of the aggregation procedure, they are related in the following way:

$$\begin{aligned} g_{31} &= h_{11} \frac{V_{-1}}{V_3} \\ g_{32} &= h_{22} \frac{V_{-2}}{V_3} \\ g_{34} &= h_{14} \frac{V_{-1}}{V_3} + h_{24} \frac{V_{-2}}{V_3}. \end{aligned} \tag{11}$$

Similarly, α_3 is not an independent rate constant, it is related to β_1 and β_2. On the other hand, the precursor–product relationships determine that $V_{-3} = V_4$, so that g_{43} is equivalent to h_{33} and α_4 is equal to β_3. The S-system representation of the reference system in Fig. 1 is thus:

$$\begin{aligned} \dot{X}_1 &= \alpha_1 X_5^{g_{15}} - \beta_1 X_1^{h_{11}} X_4^{h_{14}} \\ \dot{X}_2 &= \alpha_2 X_6^{g_{26}} - \beta_2 X_2^{h_{22}} X_4^{h_{24}} \\ \dot{X}_3 &= \alpha_3 X_1^{g_{31}} X_2^{g_{32}} X_4^{g_{34}} - \beta_3 X_3^{h_{33}} \\ \dot{X}_4 &= \beta_3 X_3^{h_{33}} - \beta_4 X_4^{h_{44}}. \end{aligned} \tag{12}$$

Values for the S-system parameters in the reference system

The value of the S-system parameters for the example system are computed from the kinetic description (see Table 3). In each case, we identify the appropriate flux (V_i or V_{-i}) with its kinetic rate-law and we obtain the kinetic orders by derivation and evaluation at the operating state of interest (Voit & Savageau, 1987; Sorribas & Savageau, 1989*a*). These values provide an appropriate reference for evaluating the suggested estimation procedure.

Estimation of S-system parameters from simulated experiments

As stated above, to estimate the difference $(g_{ik} - h_{ik})$ we measure the initial change in X_i after a perturbation in X_k. The number of perturbation experiments required is determined by the system structure through the non-zero kinetic orders that need to be estimated. In Table 2 we present the estimated initial rates of change in each dependent metabolite after a 15%, 25% and 100% perturbation in the appropriate steady-state values. As an example, the set of simulated data used for computing the initial rate of change in X_1 after a perturbation in X_4 is shown in Fig. 3. The value of c_1 corresponding to each condition is computed using polynomial regression [eqns

TABLE 3

Parameters estimated from simulated experiments

Parameter	Reference value	Estimated		
		15%	25%	100%
g_{31}	0·542	0·525	0·515	0·457
		(0·511)	(0·501)	(0·442)
g_{32}	0·303	0·302	0·299	0·279
		(0·289)	(0·288)	(0·271)
g_{34}	−0·276	−0·267	−0·259	−0·211
		(−0·258)	(−0·251)	(−0·206)
g_{43}	0·500	0·471	0·451	0·0339
		(0·458)	(0·440)	(0·333)
g_{15}	0·670	0·640	0·580	0·499
g_{26}	0·833	0·813	0·800	0·750
h_{11}	0·813	0·787	0·772	0·685
h_{14}	−0·187	−0·187	−0·184	−0·159
h_{22}	0·909	0·907	0·896	0·838
h_{24}	−0·455	−0·427	−0·408	−0·314
h_{33}	0·500	0·471	0·451	0·339
h_{44}	0·500	0·476	0·456	0·339
α_1	0·0737	0·0818	0·103	0·142
α_2	0·0192	0·0208	0·0219	0·0266
α_3	0·0895	0·900	0·905	0·931
		(0·916)	(0·919)	(0·946)
α_4	1·50	1·50	1·50	1·50
β_1	0·648	0·660	0·665	0·694
β_2	0·252	0·248	0·248	0·248
β_3	1·50	1·50	1·50	1·50
β_4	1·06	1·08	1·09	1·19

For g_{31}, g_{32}, g_{34} and g_{43} the values without parentheses are computed from product–precursor relationships. The values in parentheses are computed from perturbation experiments.

(7) and (8)]. Once these rates are computed, the parameter values are estimated by using eqn (9) (Table 3).

Influence of experimental error

Figure 4 shows how the experimental error in determining the actual concentration at each time point affects the estimation of the kinetic orders of synthesis and degradation of X_1. As expected, the introduction of experimental error results in inferior precision in the estimated parameter values. This is particularly significant when the initial rate of change (the slope of the time course at $t=0$) is low. In this case, measurement errors can lead to unrealistic values of the slope at $t=0$, especially for small perturbations. An example is the response of X_1 to a change in X_4 (Fig. 3). As shown in Fig. 4(c), the estimated value of h_{14} ranges from negative to positive with a mean far from its actual value when a 15% perturbation in X_4 is considered. In this case, the accuracy of the estimates is highly compromised by the fact that a low value of h_{14} implies a poor initial response when considering a small perturbation. In consequence, a slightly better result can be obtained if we use a 25% perturbation

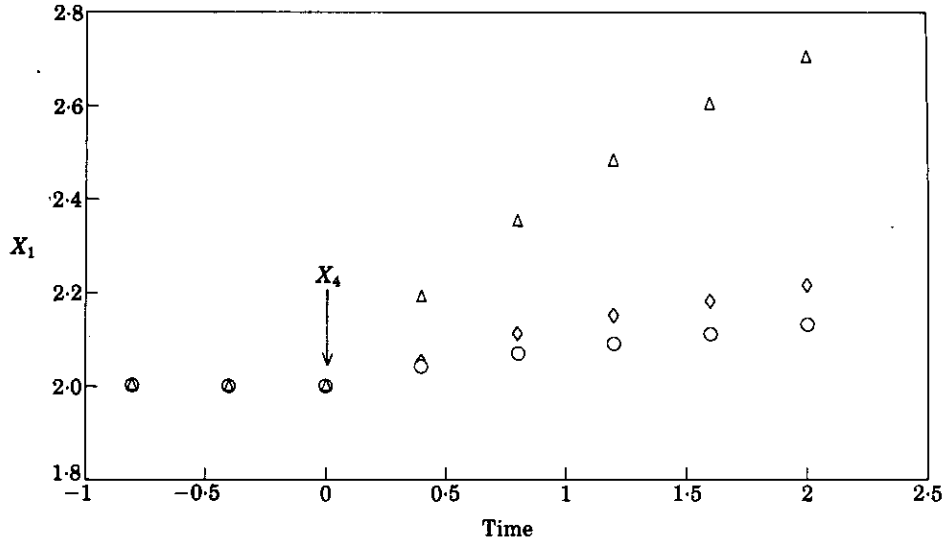

FIG. 3. Response of X_1 to a perturbation in X_4. The change in concentration of X_1 after a percent perturbation in X_4 [(○)+15%; (◇)+25% and (△)+100%] is computed by using the kinetic equations presented in the Methods. These data are error free. The value of the initial rate of change in X_i, corresponding to each perturbation is obtained from a polynomial fit to each data set as presented in the Theory section.

to compute the initial rate of change [Fig. 4(d)]. However, the accuracy in estimating the actual value of h_{14} is still poor.

To overcome this problem, we shall consider repeated measurements for each time point. It is expected that such a procedure leads to a more precise estimation of the target parameters if the method has a consistent behavior. In Fig. 5 we show the results from simulated experiments with the same error structure and three measurements at each point. The improvement in the estimated values of h_{14} is evident both in experiments with a 15% perturbed value of X_4 [Fig. 4(b)] and in experiments with a 25% perturbed value of X_4 [Fig. 4(d)]. Hence, for practical purposes, repeated measurements should be considered in order to improve accuracy, especially when the obtained values are close to zero.

Characterization of the reference system using the estimated parameters

Logarithmic gains

In Table 4, the logarithmic gains of dependent concentrations are shown. We obtain a similar characterization when we use the actual values of the S-system parameters, the values obtained in a 15% perturbation experiment, and the parameters obtained from a 25% perturbation experiment (see values in Table 3). Although the estimated parameters are not exactly equal to the true values, the characterization of the response of the system to a change in an independent variable appears to be

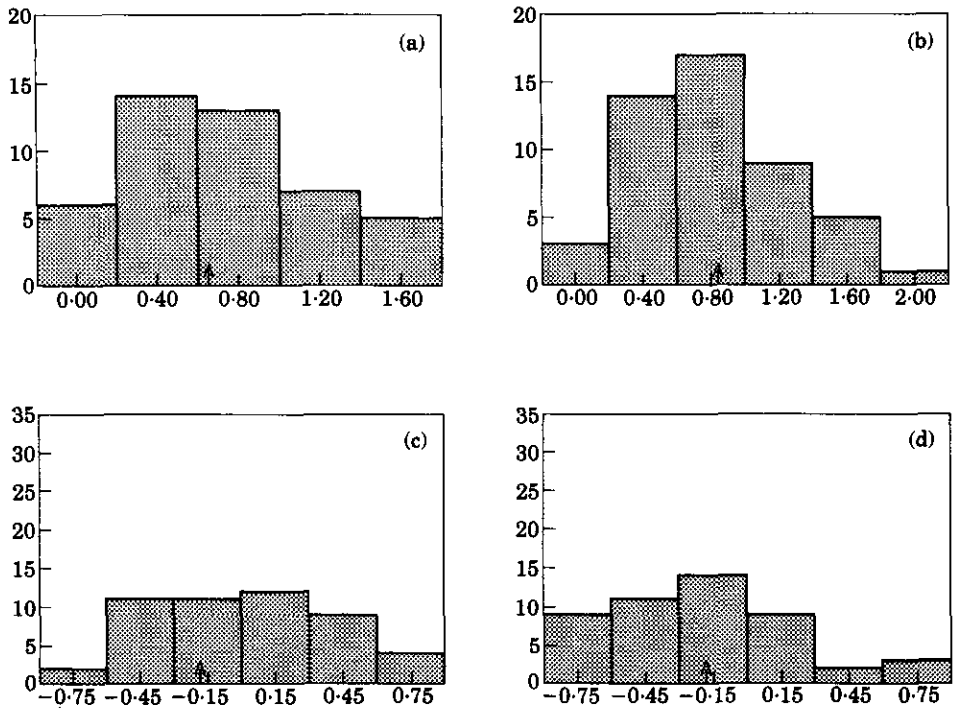

FIG. 4. Influence of experimental error on the estimation procedure. The parameters concerning the synthesis and degradation of X_1 are estimated from a set of 50 simulated experiments in which the data are modified by adding statistical noise with zero mean and standard deviation equal to 2·5% of the actual value of the corresponding metabolite. Perturbation conditions: (a) 15% increase in X_5; (b) 15% increase in X_1; (c) 15% increase in X_4; (d) 25% increase in X_4. The mean and the standard error of the mean of the 50 estimated values are: (a) $\bar{x}$: 0·756, SEM: 0·066; (b) $\bar{x}$: 0·782, SEM: 0·070; (c) $\bar{x}$: 0·003, SEM: 0·061; (d) $\bar{x}$: −0·235, SEM: 0·063. The reference values of the parameters, indicated by an arrow, are: (a) g_{15}: 0·670; (b) h_{11}: 0·813; (c) and (d) h_{14}: −0·187 (Table 3).

accurate enough for practical purposes. This accuracy is an indication that the steady-state characterization is remarkably robust to fluctuations in the values of the kinetic order set.

Rate-constant sensitivities

Table 5 shows the computed rate-constant sensitivities for the set of independent parameters. The computed sensitivities are quite similar, both for the estimated kinetic-orders and for the actual set of parameters. As in the case of the logarithmic gains, the steady-state characterization is accurate enough using the estimated parameter set.

Kinetic-order sensitivities

Table 6 shows the kinetic-order sensitivities for the dependent metabolites. The set of independent parameters is determined after considering the aggregation

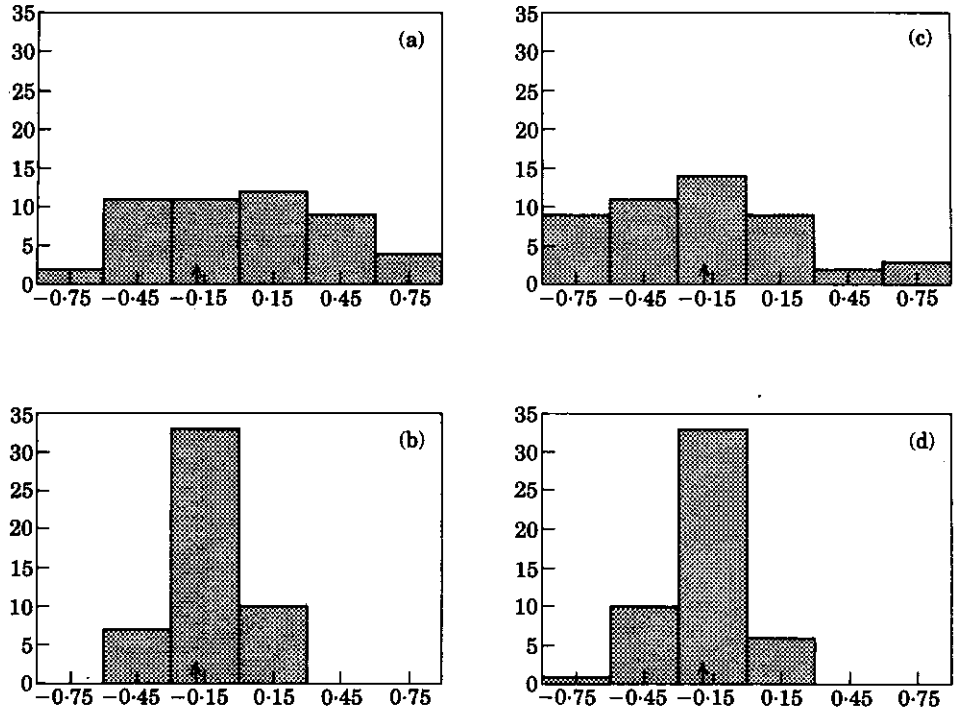

FIG. 5. Improvement of the estimation by using repeated measurements. The effect of using repeated measurements on the estimation of h_{14} is investigated. In each case, we represent the estimated parameter value of 50 simulated experiments. (a) 15% perturbation in X_4 with a unique measurement; (b) 15% perturbation in X_4 with three measurements at each time point; (c) 25% perturbation in X_4 with a unique measurement; (d) 25% perturbation in X_4 with three measurements at each time point. The mean and the standard error of the mean of the 50 estimated values are: (a) $\bar{x}$: 0·003, SEM: 0·061; (b) $\bar{x}$: −0·235, SEM: 0·063; (c) $\bar{x}$: −0·197, SEM: 0·026; (d) $\bar{x}$: −0·126, SEM: 0·025. The reference value of h_{14}, indicated by an arrow, is −0·187 (Table 3).

TABLE 4

Steady-state characterization of the reference system

Independent variable		Dependent variable							
		X_1	X_2	X_3	X_4	V_1	V_2	V_3	V_4
X_5	Refer.	1·03	0·444	0·889	0·889	0·667	0·00	0·444	0·444
	15%	1·03	0·422	0·906	0·897	0·640	0·00	0·427	0·427
	25%	0·953	0·386	0·858	0·848	0·580	0·00	0·387	0·387
X_6	Refer.	0·128	1·19	0·555	0·555	0·00	0·833	0·278	0·278
	15%	0·135	1·16	0·575	0·569	0·00	0·813	0·271	0·271
	25%	0·140	1·16	0·592	0·585	0·00	0·800	0·267	0·267

Logarithmic gains computed from the estimated parameter values in Table 3 (when necessary, precursor–product derived values are considered).

TABLE 5

Steady-state characterization of the reference system

Independent rate constant		Dependent variables			
		X_1	X_2	X_3	X_4
α_1	Refer.	1·53	0·659	1·33	1·33
	15%	1·60	0·659	1·42	1·40
	25%	1·64	0·666	1·48	1·46
α_2	Refer.	0·152	1·43	0·666	0·666
	15%	0·166	1·43	0·708	0·700
	25%	0·174	1·45	0·739	0·739
$\alpha_4=\beta_3$	Refer.	0·000	0·000	−2·00	0·000
	15%	0·000	0·000	−2·12	0·000
	25%	0·000	0·000	−2·22	0·000
β_1	Refer.	−1·23	0·000	0·000	0·000
	15%	−1·27	0·000	0·000	0·000
	25%	−1·30	0·000	0·000	0·000
β_2	Refer.	0·000	−1·10	0·000	0·000
	15%	0·000	−1·10	0·000	0·000
	25%	0·000	−1·12	0·000	0·000
β_4	Refer.	−0·456	−0·988	0·000	−2·00
	15%	−0·499	−0·989	0·000	−2·10
	25%	−0·523	−0·999	0·000	−2·19

Rate-constants sensitivities of dependent concentrations computed from the estimated parameter values in Table 3 (when necessary, precursor–product derived values are considered).

procedures and the precursor–product relationships [eqn (10)]. As in the preceding cases, the system characterization is remarkably robust to fluctuations in the set of parameters.

Dynamic behavior

An important advantage of using the S-system representation is its capability of representing the dynamic behavior of the target system. To show this feature, we compare the time response after a perturbation in the operating value of X_5 (Fig. 6). For each set of parameter values, there is good agreement between the behavior obtained from the kinetic equations and the estimated S-system equations. Again, although the estimated parameter values are not exactly equal to the reference values, the picture of the systemic behavior is remarkably close to the one we would obtain for a representation based on kinetic equations.

A brief insight into the properties of the reference system

In studying a real metabolic problem, the ultimate goal is to reach understanding on the properties of the target pathway. However, understanding means different things to different scientists. For some of them, understanding means being able to predict the future behavior of the system; for others, devising strategies that lead to the formulation of general rules in biochemistry; for yet another group, understanding means obtaining some numbers that characterize the regulatory properties of the system studied; finally,

TABLE 6

Steady-state characterization of the reference system

Independent kinetic orders		Dependent variables			
		X_1	X_2	X_3	X_4
g_{15}	Refer.	4·01	1·72	3·49	3·49
	15%	4·01	1·65	3·54	3·50
	25%	3·72	1·51	3·35	3·31
g_{26}	Refer.	0·494	4·65	2·16	2·16
	15%	0·529	4·55	2·25	2·22
	25%	0·545	4·53	2·31	2·28
h_{11}	Refer.	−0·691	0·00	0·00	0·00
	15%	−0·692	0·00	0·00	0·00
	25%	−0·689	0·00	0·00	0·00
h_{14}	Refer.	0·158	0·00	0·00	0·00
	15%	0·164	0·00	0·00	0·00
	25%	0·165	0·00	0·00	0·00
h_{22}	Refer.	0·00	−1·09	0·00	0·00
	15%	0·00	−1·09	0·00	0·00
	25%	0·00	−1·10	0·00	0·00
h_{24}	Refer.	0·00	0·341	0·00	0·00
	15%	0·00	0·325	0·00	0·00
	25%	0·00	0·317	0·00	0·00
h_{33}	Refer.	0·00	0·00	0·002	0·00
	15%	0·00	0·00	0·003	0·00
	25%	0·00	0·00	0·004	0·00
h_{44}	Refer.	−0·158	−0·341	0·00	−0·692
	15%	−0·164	−0·325	0·00	−0·690
	25%	−0·165	−0·317	0·00	−0·696

Kinetic-order sensitivities of dependent concentrations computed from the estimated parameter values in Table 3 (when necessary, precursor–product derived values are considered).

for the least demanding group, it means just having a broad picture of the processes involved.

As an example in providing answers to these kind of questions in a specific situation, we highlight several features that may be of interest for evaluating the performance of our reference system at the conditions considered. This evaluation may provide valuable insight, for instance, in suggesting which kind of manipulation would lead to an improvement of the system performance (for example, optimization of X_4 production by manipulating the substrates or by modifying the underlying processes by means of biotechnological methods).

From the numerical characterization of this system (Tables 4–6), we emphasize the following features:

(i) The increase in X_4 after a change in X_5 is greater than after a change in X_6 (Table 4). Hence, if we are interested in manipulating this system to raise the production of X_4, we should focus our attention in X_5.

(ii) The production of X_3 from X_1 is a good candidate for trying to modify the rate-constant in order to produce an increase in the steady-state level of X_4

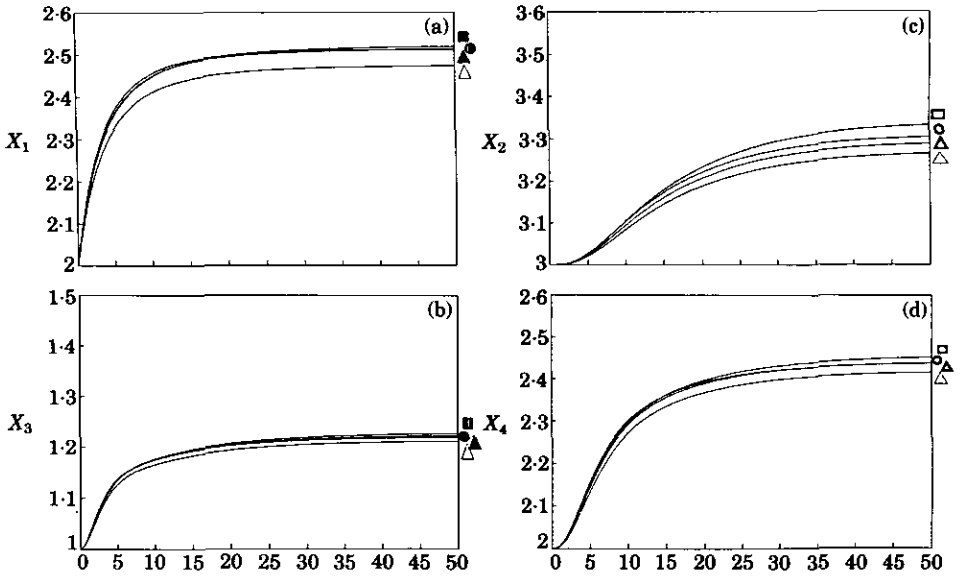

FIG. 6. Comparison of the dynamic response after a change in an independent variable. The predicted dynamic responses are compared with different parameter sets. (■) kinetic equations; (⊖) reference S-system parameters; (▲) estimated S-system in 15% perturbation experiments; (△) estimated S-system parameters in 25% perturbation experiments.

(Table 5). A change in this rate-constant is equivalent to changing the amount of enzyme catalyzing this reaction if its rate is proportional to the enzyme concentration. However, a change in α_1 results in an important increase in the concentration of all the dependent metabolites, which could be considered as an undesirable side effect. Thus, although an increase in α_2 produces a lower increment in X_4 (Table 5), the corresponding increase in the other metabolites is also low, especially in X_1. It is important to be able to evaluate these comparative responses in order to suggest the best decision.

(iii) The system is sensitive to changes in the kinetic-order parameters (Table 4). Clearly, the most influential parameters are g_{15} and g_{26}. This was expected because the system is essentially irreversible and the flux is fully determined by the processes of synthesis of X_1 and X_2. An increase in the demand of X_4, which can be viewed as an increase in h_{44}, has a negative influence on the logarithmic gain of this metabolite in response to an increase of the system substrates, although this effect is the same in both logarithmic gains. This effect is computed after obtaining the logarithmic gains algebraically from the steady-state equations (results not shown; see Savageau, 1976).

A PRACTICAL RECIPE FOR CHARACTERIZING A METABOLIC PATHWAY BY USING THE ESTIMATION PROCEDURE DEVELOPED IN THIS PAPER

The approach developed in this paper allows the characterization of a metabolic pathway from measurements *in vivo*. As a practical recipe for its application to a

specific problem, we recommend the following steps, which may provide guidelines for using the S-system methodology in characterizing a specific metabolic pathway:

(i) Draw a scheme of the target system. Include all the regulatory signals and define which variables are considered to be independent.

(ii) Write the S-system representation following the scheme and according to the rationale indicated in this paper (see Results).

(iii) Examine, for each dependent variable, how many variables affect its synthesis and/or degradation. This gives the clue for planning the perturbation experiments.

(iv) Measure the steady-state values of the system variables. This is the operating steady-state at which the system will be characterized.

(v) Measure the appropriate time courses needed to characterize the a_{ik} parameters according to point (iv). Use repeated measurements to increase accuracy.

(vi) Compute the values of g_{ik} and h_{ik} from a_{ik}. Use the precursor–product relationships when needed. Once these parameters are obtained, compute the appropriate rate-constants from the steady-state values of the variables of the system.

(vii) Use the program ESSYNS (or perform the appropriate algebraic operations) to obtain the characterization of the system (logarithmic-gains and parameter sensitivities).

(viii) Perform simulated experiments by using the S-system equations and the estimated parameters.

(ix) With the information obtained from points (vii) and (viii), the properties of the considered operating steady-state can be discussed.

(x) If we are interested in a more general result, a theoretical analysis of the properties of the system can be performed. This is achieved by symbolic algebra and may include considerations of optimization after defining criteria for functional effectiveness (see Savageau, 1976; Irvine & Savageau, 1985*a*, *b*; Irvine, 1991, for examples).

Discussion

Among the experimental procedures devised for characterizing a biochemical pathway through the use of the power-law formalism, there is no single estimation method that can account for the needed parameter set in any condition. Most of the available techniques focus on measuring some steady-state properties upon manipulating the system by adding external elements. Some of these methods include genetic manipulation and the use of irreversible inhibitors which must be specific for a single enzyme in the pathway. Although these approaches provide valuable results in specific cases, often the requirements for their application limit their practical usefulness. Other methods are based on measurements of the isolated components *in vitro*. These must be considered with caution because the original structure and relationships present in the intact system may not be preserved. Yet, other possibilities include enzyme titration, multiple steady-state measurements and so on. However, in considering

these alternative procedures almost no effort has been dedicated to exploit the information included in the dynamic response of the system.

In this situation, an estimation method able of utilizing the dynamic data can help in properly characterizing a given metabolic pathway if the required data are obtainable. The estimation procedure presented in this paper provides such a tool through the use of an appropriate analytical approach: the S-system representation. The required measurements, transient responses after metabolite perturbation, are now widely available with a number of techniques and they are accurate enough for the required parameters to be properly identified. Our results show that the performance of the suggested estimation method, validated through the analysis of a reference system in simulated experiments, is good enough to be used in experimental studies. As a limitation, we shall consider the fact that in a given situation it could not be easy to properly manipulate the involved metabolites. Although this limitation can be overcome with new experimental devices, it can be an important obstacle for obtaining the complete set of parameters. In such a case, we should consider the use of alternative methods based in steady-state measurements.

In any case, an important advantage of using the approach developed in this paper is that the experimental effort needed to provide the appropriate data can be considerably less than the effort needed if we approach the problem in a more classical way, say by means of kinetic experiments. In this case, a large set of kinetic experiments would be necessary to identify both the mechanism and each kinetic parameter of each isolated enzyme. In contrast, if we approach the problem from an integrative point of view, as is the case with the S-system representation, direct measurements on the intact system can be performed and the parameters can be determined with much less experimental effort. As has been stated before, the S-system equations are not an alternative to the mechanistic rate laws for isolated enzymes, they are an alternative to the description of the whole system. Hence, the S-system parameters refer to the global description of the target system and the estimation procedure suggested in this paper is aimed at this systemic goal. Previous experience in using the S-system approach and our present results demonstrate that S-systems provide representations that often are sufficiently accurate in comparison with the more elaborate kinetic approach. Hence, the S-systems can be considered appropriate standard representations for intact biochemical systems, both in terms of simplicity and accuracy. The results shown in this paper also reveal how some of the goals in understanding a metabolic system can be reached when we analyze a specific pathway within the framework of S-systems. Although some of the conclusions on the properties of the reference system could be seen as quite intuitive, none of these can be reached without an appropriate analytical tool allowing for numerically evaluating the system's characteristics. Although we have concentrated on three specific points, the results in Tables 4–6 and the dynamic example (Fig. 6) display the ability of the S-system methodology to lead to a complete characterization of the target system.

The S-system approach is a well-defined framework for analyzing intact metabolic pathways. Its application to different problems has led to important conclusions based on considerations on design principles and metabolic effectiveness. However,

its application to specific metabolic situations has been limited, and this has resulted in a restricted spread of this technique among biochemists. We hope that the method suggested in this paper can help in bridging the gap between the theoretical results and the experimental measurements.

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