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Enzyme kinetics and metabolic control

A method to test and quantify the effect of enzymic properties on metabolic variables

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It is usual to study the sensitivity of metabolic variables to small (infinitesimal) changes in the magnitudes of individual parameters such as an enzyme concentration. Here, the effect that a simultaneous change in all the enzyme concentrations by the same factor α (Co-ordinate-Control Operation, CCO) has on the variables of time-dependent metabolic systems is investigated. This factor α can have any arbitrary large value. First, we assume, for each enzyme measured in isolation, the validity of the steady-state approximation and the proportionality between reaction rate and enzyme concentration. Under these assumptions, any time-invariant variable may behave like a metabolite concentration, i.e. $S_\alpha = S_r$ (S-type), or like a flux, i.e. $J_\alpha = \alpha J_r$ (J-type). The subscripts r and α correspond to the values of the variable before and after the CCO respectively. Similarly, time-dependent variables may behave according to $S_\alpha(t/\alpha) = S_r(t)$ (S-type) or to $J_\alpha(t/\alpha) = \alpha J_r(t)$ (J-type). A method is given to test these relationships in experimental systems, and to quantify deviations from the predicted behaviour. A positive test for deviations proves the violation of some of the assumptions made. However, the breakdown of the assumptions in an enzyme-catalysed reaction, studied in isolation, may or may not affect significantly the behaviour of the system when the component reaction is embedded in the metabolic network.

1. INTRODUCTION

Enzymologists have been studying the kinetic properties of isolated enzyme-catalysed reactions for many years. In the great majority of the reactions the steady-state rate is proportional to the total enzyme concentration. Many exceptions to this property are reported, and this fact is often connected with the existence of subunit associations (see, e.g., Kurganov, 1978). Another common characteristic of most enzyme assays is that the rate remains constant during the early course of the reaction. Some examples are, however, known where the 'initial' rate increases (lag) or decreases (burst) in time (see, e.g., Neet & Ainslie, 1980). In these cases the steady-state assumption for the concentrations of the different enzyme forms is violated.

Enzyme-catalysed reactions are the building blocks of metabolism, and the knowledge of their kinetic features is an important step towards understanding how metabolic networks behave. Nevertheless, we must note that these individual reactions are part of a system where the components influence each other in intricate ways. In metabolic systems the metabolite concentrations are not held constant, as in traditional enzyme kinetic assays. In these systems the rates (i.e. fluxes) affect the metabolite concentrations, and these in turn affect the rates (Kacser, 1987). We must conclude that all the components contribute to the system behaviour to some extent. However, if a component is replaced by a different one, are the properties of the system significantly changed? Or, to particularize the question, is a particular kinetic feature of an enzyme-catalysed reaction (e.g. rate non-proportional to enzyme concentration) relevant to the behaviour of a metabolic variable when the enzyme concentration is changed? As we show in the present paper, the existence of strong deviations from proportionality between rate and enzyme concentration (in a traditional assay) may be almost irrelevant when the enzyme concentration is changed within a metabolic network, whereas in other cases small deviations from proportionality (measured in isolation) may be greatly amplified in the system. Similar conclusions apply to enzymes that exhibit lags or bursts. Furthermore, we show how the quantitative importance of the effects that these kinetic properties of enzymes have on a metabolic variable may be experimentally determined. The relationships and methods developed in the present paper apply to time-dependent metabolic systems. They enable one to analyse the properties of the instantaneous values of a time-dependent variable as well as the properties of the time-invariant variables that may be defined from the time course (e.g. steady-state values).

2. PARAMETERS AND VARIABLES

A metabolic system is, basically, a network constituted of molecules, x_i, 'connected' by chemical reactions. This is usually represented by a 'static' metabolic map, but in our treatment we wish to study some quantitative aspects of its dynamical behaviour. The rates of interconversion between each pair of molecules are given by the rate laws, v_k. These may be functions of the concentrations, x_i, involved (free metabolites, free enzymes, enzyme–metabolite complexes, enzyme–enzyme complexes etc.), temperature, pressure, pH, ionic strength etc.

The 'parameters' of the system are the quantities that can be manipulated independently of each other. Once their values are fixed at the initial point of time, they remain constant during the whole interval of time that the system is studied. In what follows, we consider as parameters the total concentration of each enzyme (free plus complex forms) and physicochemical quantities such as temperature and pressure. The fluxes or free concentrations that act as inputs of the system (e.g. sources and sinks of matter and external effectors) are either held constant or changed in time in

Abbreviations used: CCO, Co-ordinate-Control Operation; D-plot, Direct co-ordinate-control plot; R-plot, Rescaling co-ordinate-control plot; RPS-plot, Reference-Point Sensitivity co-ordinate-control plot; PPS-plot, Point-to-Point Sensitivity co-ordinate-control plot.

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some pre-determined way (an example of the latter situation is given in Markus *et al.*, 1984).

The 'variables' of the system are the quantities whose values depend on the values of the parameters. We may define two types of variables, namely time-dependent and time-invariant, whether their magnitudes do or do not change with time respectively. The instantaneous concentrations x_i, mentioned above, and other quantities that are functions of these concentrations (e.g. fluxes) are time-dependent variables. The successive values that they take in time depend on the values to which the parameters and initial concentrations are set at the initial time. Examples of time-invariant variables are the stable steady-state concentrations and fluxes, the transition time of a metabolite (Heinrich & Rapoport, 1975) or a pathway (Easterby, 1981, 1986) to a stable steady state, the period and amplitude of variables that exhibit sustained oscillations (Hofmann *et al.*, 1985; Goldbeter & Moran, 1987; Mizraji *et al.*, 1988) and the maximum Lyapunov exponent that characterizes a chaotic regime (Hess & Markus, 1987).

First, we centre the attention on the time-dependent variables x_i. The change of each x_i with time, dx_i/dt, may be written as the balance of all the rates, v_k, that affect its concentration directly:

$$\frac{dx_i}{dt} = \sum_{k=1}^{p} n_{ik} \cdot v_k, \qquad i = 1, \ldots, q \tag{1}$$

Here n_{ik} is the stoichiometric coefficient of the molecule x_i in the reaction k. It is positive, negative or zero if x_i is produced, consumed or not altered directly by the rate v_k respectively. For a given set of values of the parameters and initial concentrations, the solutions of eqns. (1) constitute the time courses of the concentrations: $x_i(t)$, $i = 1, \ldots, q$. Introducing these $x_i(t)$ into the rate laws v_k, we obtain the time courses of the fluxes: $J_k(t)$, $k = 1, \ldots, p$.

The values of the time-invariant variables may be obtained from the time course of the time-dependent variables. For example, when a system settles to a stable steady state, an estimate of the steady-state values of metabolite concentrations and fluxes may be obtained from the time courses, by waiting a 'long enough' period of time. In a system that exhibits sustained oscillations in time, the period of oscillation (the interval of time between two consecutive maxima in the time course) is such a time-invariant variable.

3. THE CO-ORDINATE-CONTROL OPERATION (CCO)

It has been traditional to investigate systems by a sensitivity analysis of the variables with respect to specific parameters. Thus control analysis (Kacser & Burns, 1973; Heinrich & Rapoport, 1974) considers the responses of metabolic concentrations and fluxes to modulations of any one of the parameters of the system. Some progress has been made to use this approach to detect deviations from the assumption of proportionality between rate and enzyme concentration in steady-state systems (Kacser *et al.*, 1990; Sauro & Kacser, 1990). In what follows, a different method from modulating individual parameters is described. It applies to time-dependent systems and has the advantage of not being restricted to small (infinitesimal) changes.

Changes in the values of the parameters affect, to various degrees, the values of the variables (control of variables by parameters). For a time-dependent variable, one may define a 'reference time course' generated by a chosen set of values of the parameters, the 'reference parameter values'. If one or more of the reference parameter values are altered at the initial time, the resulting time course may be significantly different from the reference one. In what follows, we study the control of the variables by a particular group of parameters, namely the total enzyme concentrations, E_j. We assume that all the m enzyme concentrations are simultaneously changed by the same arbitrary factor α (not necessarily ≈ 1). If $E_{j,r}(j = 1, \ldots, m)$ are the values of the enzyme concentrations that generate the reference time course, then the new time course is obtained by using enzyme concentrations $E_{j,\alpha}(j = 1, \ldots, m)$ given by:

$$E_{j,\alpha} = \alpha E_{j,r} \tag{2}$$

We call this equal and simultaneous change in all the enzyme concentrations: the Co-ordinate-Control Operation (CCO) (briefly introduced in Acerenza *et al.*, 1989; Acerenza, 1990). The subscripts r and α are used to indicate the value of a parameter or variable before (reference) and after the CCO is applied respectively. We use this operation throughout the following treatment. This approach reveals certain simple properties of time-dependent metabolic systems, when some assumptions are made (see below). Furthermore we suggest how the resulting relationships, and hence the assumptions made, may be experimentally tested in reconstituted systems or biological extracts. We discuss the practical problems associated with attempting to apply a CCO in a subsequent section.

4. ASSUMPTIONS

We now make some assumptions concerning the properties of the metabolic system. These are used to derive some theoretical consequences of the CCO in sections 5 and 6. The analysis of cases where there is a breakdown of the assumptions is considered in sections 9–11.

In the general case (see section 2) the q concentrations x_i that appear in eqns. (1) may be classified in two groups: n free metabolite concentrations, S_i, and $q-n$ enzyme concentration in their different forms (free or combined with metabolites), C_i. If the steady-state approximation for the concentrations C_i is plausible, then $dC_i/dt = 0$ for each C_i (Segel, 1988). Applying these conditions to eqns. (1), the reduced resulting system of differential equations:

$$\frac{dS_i}{dt} = \sum_{j=1}^{m} n_{ij} \cdot v_j, \qquad i = 1, \ldots, n \tag{3}$$

involves only the free metabolite concentrations as variables. In addition, we assume that the rates v_j are proportional to the corresponding total enzyme concentrations E_j:

$$v_j = E_j \cdot f_j, \qquad j = 1, \ldots, m \tag{4}$$

where f_j are functions of the concentrations S_i and parameters, and are independent of enzyme concentrations and time. The Michaelis–Menten rate equation, for example, fulfils eqn. (4).

Applying the CCO to a metabolic system whose rates are given by eqn. (4), the resulting rates, $v_{j,\alpha}$, are related to the reference rates, $v_{j,r}$ (see eqn. 2), as follows:

$$v_{j,\alpha} = \alpha v_{j,r}, \qquad j = 1, \ldots, m \tag{5}$$

Then, under the assumptions described by eqns. (3) and (4), the first important consequence of the CCO is to multiply each term of the right-hand member of eqns. (3) by the same factor α. It is important to note that, if matter is introduced into the system via one or more constant input fluxes, these should also be modified according to eqn. (5) when the CCO is applied. However, any constant (input) concentrations, if present, should not be modified when the rate that transforms them is given by eqn. (4). The discussion of the case where the inputs are changed in time, by the experimentalist, is postponed to section 5.

5. CO-ORDINATE CONTROL OF TIME-DEPENDENT VARIABLES

In this section we outline some consequences of the CCO, related to the control of time-dependent variables. Some mathematical details of this treatment are given in Acerenza *et al.* (1989).

Combining eqns. (3) and (5), we obtain the relationship between the derivative of S_i with respect to time after and before (reference) the CCO:

$$\left(\frac{dS_i}{dt}\right)_\alpha = \alpha\left(\frac{dS_i}{dt}\right)_r \quad (6)$$

The only effect of a simultaneous change in all the enzyme concentrations by a factor α is to make the metabolite concentrations change at a rate that is α times the original one. Then, the CCO is equivalent to a change in the time scale of the time courses of the metabolite concentrations. For each time t_r of the reference time courses [the reference time courses are the functions of time, $S_i(t)$, obtained with the reference parameter values] there exists one time t_α, in the new time courses, at which all the metabolite concentrations have the same values as in the reference state at time t_r. The value of t_α is given by:

$$t_\alpha = \frac{t_r}{\alpha} \quad (7)$$

and hence:

$$S_{i,\alpha}(t_r/\alpha) = S_{i,r}(t_r) \quad (8)$$

From eqns. (5) and (8) we obtain the analogous relationship for the fluxes:

$$J_{j,\alpha}(t_r/\alpha) = \alpha J_{j,r}(t_r) \quad (9)$$

Eqns. (8) and (9) tell us that, when the CCO is applied to a time-dependent metabolic system, which satisfies the assumptions made in eqns. (3) and (4), the instantaneous values of the metabolite concentrations are 'shifted' from the time t to t/α, while the instantaneous values of the fluxes are multiplied by the factor α and 'shifted' from t to t/α. It is important to note that, if matter is introduced into the system via concentrations or fluxes that change in time, these inputs should be altered in the same way as shown by eqns. (8) and (9) respectively when the CCO is applied.

An immediate consequence of eqn. (8) is that if one plots the metabolite concentrations after the CCO against α multiplied by time the resulting curve should coincide with the reference time course (see Figs. 1*a* and 1*b*). This result is used below in section 8. [A similar procedure is used as a test for inactivation of a single enzyme during assay (Selwyn, 1965); (see also Cornish-Bowden, 1979).]

6. CO-ORDINATE CONTROL OF TIME-INVARIANT VARIABLES

A. Co-ordinate control of time-invariant variables with dimension of time

Time-invariant variables with dimension of time, which characterize some temporal aspect of the time course of the metabolite concentrations, satisfy eqn. (7). Examples of these variables are transition times, period of oscillation and the reciprocal of the maximum Lyapunov exponent (mentioned in section 2). Then, if T_r is the value of such a time-invariant variable with dimension of time, obtained from the reference time course, after the CCO the new value of the variable, T_α, is given by:

$$T_\alpha = \frac{T_r}{\alpha} \quad (10)$$

that is a simultaneous increase (decrease) in all enzyme concentrations by a factor α causes a decrease (increase) in the value of T by the same factor.

B. Co-ordinate control of a transition to a stable steady state

If the metabolic system is one that approaches a stable steady state, after a long enough period of time the variables exhibit

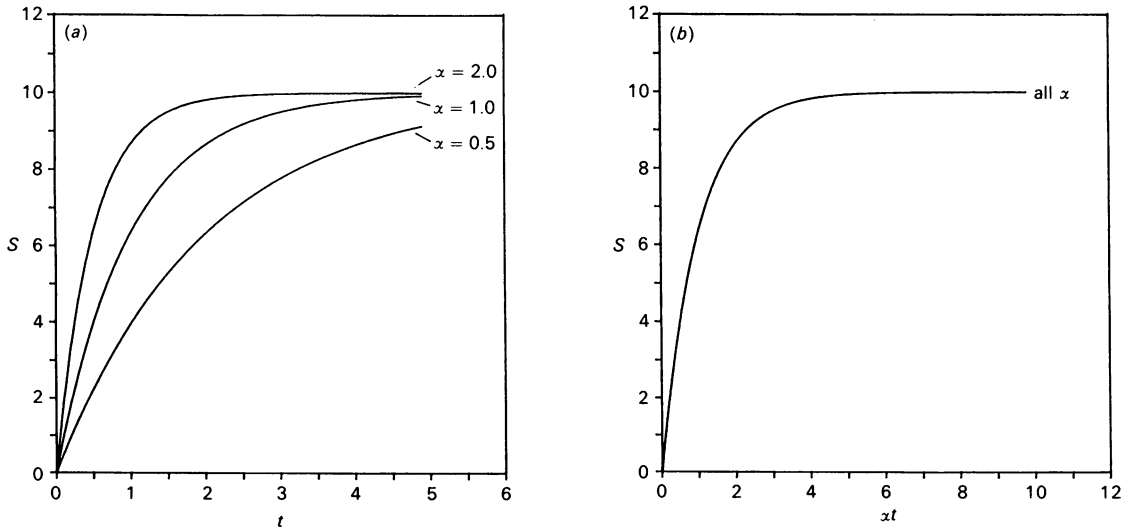

Fig. 1. Example where assumptions eqns. (3) and (4) are valid: (*a*) concentration of S versus time and (*b*) R-plot

In (*a*) we show time courses of the concentration of S (Scheme 1 in section 11) corresponding to different α values. The concentrations of X_0 and X_1 are constant. The rate laws v_1 and v_2 are proportional to the corresponding enzyme concentrations. In (*b*) we plot the same ordinate values as in (*a*), but against α multiplied by time. In this rescaling plot the three curves coincide. It should be noted that, as a result of the rescaling procedure, the curves in (*a*) corresponding to α = 0.5, 1.0 and 2.0 end in (*b*) at αt = 2.5, 5.0 and 10.0 respectively. [In Figs. 3(*a*) and 3(*b*) we show the same types of plots in a situation where assumption eqn. (4) is not valid.]

approximately constant values independent of time. Therefore eqns. (8) and (9) take the form:

$$S^{ss}_{i,\alpha} = S^{ss}_{i,r} \tag{11}$$

$$J^{ss}_{j,\alpha} = \alpha J^{ss}_{j,r} \tag{12}$$

where the superscript ss indicates steady-state values. Eqns. (11) and (12) show the effect that the CCO has on the steady-state metabolite concentrations and fluxes.

To characterize the transition between the initial conditions and the steady state one may use the transition time as defined by Easterby (1981). This is, of course, a time-invariant variable that behaves according to eqn. (10) when the CCO is applied.

C. Co-ordinate control of sustained oscillations

Here we consider the situation where the time-dependent variables (metabolite concentrations and fluxes) exhibit stable oscillations in time. In this type of behaviour, the values of the time-dependent variables repeat at constant intervals of time, T (period of oscillation). Two time-invariant variables are frequently used to characterize oscillatory phenomena, namely the period and the amplitude. The period is a time-invariant variable with dimension of time, and when the CCO is applied it behaves as shown in eqn. (10). The amplitude of oscillation (A_{S_i}) for a metabolite concentration S_i may be defined as half the difference between the maximum ($S_i^{max.}$) and minimum ($S_i^{min.}$) values: $A_{S_i} = (S_i^{max.} - S_i^{min.})/2$. The maximum and minimum values, and hence the amplitude, depend on the reference parameter values. If we apply the CCO, and wait until the system settles to a new stable oscillation, even though the value of the period is modified as described by eqn. (10), the maximum and minimum values of the metabolite concentrations are not altered (see eqn. 8):

$$\begin{aligned} S^{max.}_{i,\alpha} &= S^{max.}_{i,r} \\ S^{min.}_{i,\alpha} &= S^{min.}_{i,r} \end{aligned} \tag{13}$$

Introducing eqns. (13) into the definition of amplitude, we immediately obtain:

$$A_{S_i,\alpha} = A_{S_i,r} \tag{14}$$

i.e. the value of the amplitude is unaffected by the CCO. The fluxes J_j may be calculated by introducing the metabolite concentrations into the rate equations given in eqn. (4). If the metabolite concentrations are periodic functions of time, with period T, the corresponding fluxes are periodic functions of time with the same period. Therefore when the CCO is applied the period of these fluxes is also modified according to eqn. (10). The metabolite concentrations corresponding to the maximum and minimum fluxes are not modified, and therefore the same applies to the values of f_j at these points, introduced in eqn. (4). However, as the rates are proportional to enzyme concentration, even if the values of f_j are unaltered, the new maximum and minimum values of the flux are α times those of the reference oscillation:

$$\begin{aligned} J^{max.}_{j,\alpha} &= \alpha J^{max.}_{j,r} \\ J^{min.}_{j,\alpha} &= \alpha J^{min.}_{j,r} \end{aligned} \tag{15}$$

The amplitude of oscillation (A_{J_j}) for a flux J_j may be defined as: $A_{J_j} = (J_j^{max.} - J_j^{min.})/2$. Combining eqns. (15) with this definition, the relationship between the flux amplitude before and after the CCO is obtained:

$$A_{J_{j,\alpha}} = \alpha A_{J_{j,r}} \tag{16}$$

Besides the period and amplitude, another quantity that may be used to characterize an oscillatory regime is the mean value in a cycle. The mean value of a time-dependent variable Y in an interval of time T is defined by the expression:

$$\bar{Y}_r = \frac{\int_0^T Y_r \cdot dt}{T} \tag{17}$$

It is important to note that we evaluate the mean value in an interval of time equal to the period of oscillation. When the CCO is applied the resulting mean value can be written as follows:

$$\bar{Y}_\alpha = \frac{\int_0^{T_\alpha} Y_\alpha \cdot dt}{T_\alpha} \tag{18}$$

In the following, we discuss the relationship between $\bar{Y}_r$ and $\bar{Y}_\alpha$ when the variable is a metabolite concentration or flux that shows stable oscillations. As was mentioned above, the period of oscillation, T, satisfies eqn. (10). If Y is a metabolite concentration, eqn. (8) is fulfilled and can be written in an equivalent way: $S_{i,\alpha}(t) = S_{i,r}(\alpha t)$. Using this equation together with eqns. (10), (17) and (18), and properties of integrals, we finally obtain:

$$\bar{S}_{i,\alpha} = \bar{S}_{i,r} \tag{19}$$

In the case where Y is a flux, eqns. (9), (10), (17) and (18) are used to obtain:

$$\bar{J}_{j,\alpha} = \alpha \bar{J}_{j,r} \tag{20}$$

In other words, the simultaneous change of all enzyme concentrations by a factor α does not affect the mean values of the metabolite concentrations, but alters all the mean fluxes by the same factor (being the mean values evaluated in a period of oscillation). Eqns. (19) and (20) may be seen as equivalent to the steady-state conditions (11) and (12) when stable oscillations are considered.

7. CLASSIFICATION OF THE VARIABLES

In sections 5 and 6 we considered the effects that the CCO has on time-dependent and time-invariant variables respectively. In each one of these groups we may distinguish variables that behave like a metabolite concentration (S-type) or like a flux (J-type). Such a classification may serve as a summary of the relationships established and constitutes the basis of experimental tests.

A. Time-invariant variables

All the time-invariant variables, Y, considered in section 6, may be classified in two groups, S-type and J-type, according to the expected response when the system is subject to the CCO. We define as S-type time-invariant variables those that remain unaltered after the CCO:

$$S_\alpha = S_r \tag{21}$$

Examples of this type of variable are S_i^{ss} (eqn. 11), $S_i^{max.}$ and $S_i^{min.}$ (eqn. 13), A_{S_i} (eqn. 14) and $\bar{S}_i$ (eqn. 19). J-type time-invariant variables appear multiplied by the factor α when the CCO is applied:

$$J_\alpha = \alpha J_r \tag{22}$$

and examples of this type are J_j^{ss} (eqn. 12), $J_j^{max.}$ and $J_j^{min.}$ (eqn. 15), A_{J_j} (eqn. 16) and $\bar{J}_j$ (eqn. 20). The reciprocal of T ($1/T$) also belongs to this type (see eqn. 10).

It should be noted that, if Y is a J-type time-invariant variable, then Y/α behaves like an S-type time-invariant variable (see eqns. 21 and 22).

B. Time-dependent variables

The time-dependent variables may also be classified as *S*-type or *J*-type depending on the predicted behaviour when the CCO is applied (see section 5). The effect of the CCO on an *S*-type time-dependent variable is simply expressible as an alteration in the time scale of its time course:

$$S_\alpha(t/\alpha) = S_r(t) \quad (23)$$

The time-dependent metabolite concentrations are *S*-type variables (eqn. 8). In the case of *J*-type variables, the CCO scales simultaneously the time and the variable according to the following relationship:

$$J_\alpha(t/\alpha) = \alpha J_r(t) \quad (24)$$

Examples of *J*-type variables are the time-dependent fluxes (eqn. 9).

We therefore see that the values of an *S*-type (*J*-type) time-dependent variable, corresponding to different α and identical αt, behave like the values of an *S*-type (*J*-type) time-invariant variable. Similarly to the case of time-invariant variables, if Y is a *J*-type time-dependent variable, Y/α behaves like an *S*-type time-dependent variable.

8. TEST OF THE GENERAL RELATIONSHIPS

The data obtained from a CCO experiment may be used to test the general relationships summarized in section 7 (eqns. 21–24). Here we propose simple plots to test these relationships. Depending on whether the variables analysed are time-dependent or time-invariant, the procedures are slightly different.

A. Time-invariant variables

To test the behaviour of a time-invariant variable, Y, the basic experimental information needed is a table with the values of the variable corresponding to different α values (Y_α versus α).

The 'Direct co-ordinate-control plot' (D-plot) is simply the plot Y_α/Y_r against α. Y_r is the value of the variable when $\alpha = 1$ (reference point). The expected result of a D-plot for an *S*-type variable is a straight line where the quotients, Y_α/Y_r, are equal to 1 for all α (see eqn. 21 and Fig. 2). *J*-type variables should give a straight line, with tangent 1 (45°), that, extrapolated to α equal to zero, passes through the origin (see eqn. 22 and Fig. 2). If the D-plot is not as expected, the variable shows departures from the theoretical relationship (eqn. 21 for *S*-type and eqn. 22 for *J*-type variables). Provided that no systematic errors in applying the CCO have been introduced (see section 12), this result should be interpreted as a breakdown of the assumptions introduced in section 4. On the other hand, if the D-plot is as expected, the deviations from the assumptions, if they exist, do not contribute significantly to the behaviour of the variable when the enzyme concentrations are changed.

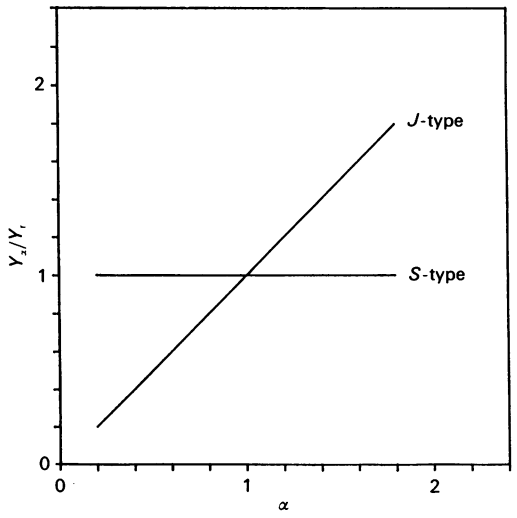

Fig. 2. D-plots, Y_α/Y_r against α, when eqns. (3) and (4) apply

The horizontal straight line is the D-plot of an *S*-type variable and the straight line of 45° corresponds to a *J*-type variable.

B. Time-dependent variables

To test eqns. (23) and (24) the experimental information needed is the time courses for different values of α, namely $Y_\alpha(t)$. On the basis of the properties of time-dependent variables, discussed in section 7, we suggest to plot the data in a 'Rescaling co-ordinate-control plot' (R-plot). The R-plot for *S*-type variables is $S_\alpha(t)$ against αt. If the variable behaves according to eqn. (23), the plots for different α should appear superimposed on the reference curve ($\alpha = 1$) (see Fig. 1). Similarly, in the case of *J*-type variables the R-plot is $J_\alpha(t)/\alpha$ against αt. As a consequence of eqn. (24), the curves corresponding to the different time courses $J_\alpha(t)$ should appear superimposed on the reference time course $J_r(t)$ (where $\alpha = 1$) in the R-plot (not shown). If such an R-plot does not give coincident curves, this would be an indication that the assumptions made in section 4 are not fulfilled, and that these deviations contribute significantly to the behaviour of the system variable when the enzyme concentrations are modified.

As was mentioned in section 7, the values of an *S*-type (*J*-type) time-dependent variable, corresponding to different α and identical αt, behave like the values of an *S*-type (*J*-type) time-invariant variable. Therefore a D-plot may be constructed with these values.

What we have called the R-plot, for *S*-type time-dependent variables, is similar to a test used to detect inactivation of an enzyme during assay (Selwyn, 1965).

9. BREAKDOWN OF THE ASSUMPTIONS

The relationships derived in sections 5 and 6, and summarized in section 7, are based on the assumptions introduced in eqns. (3) and (4). If the steady-state approximation for the different forms of the enzyme concentrations is not valid or the rates are not proportional to the corresponding enzyme concentrations, the system variables may exhibit significant deviations from the predicted behaviour (eqns. 21–24) when the CCO is applied. Here we enumerate some enzyme mechanisms that are known to violate those assumptions and, when embedded in a metabolic network, are potential generators of deviations.

Many proteins described in the literature have a quaternary structure. Depending on the experimental conditions, more than one polymeric form may coexist in significant amount. If a protein with catalytic function shows these structural features, it constitutes a source for the generation of rate laws that do not behave as eqn. (4) (see, e.g., Kurganov, 1978). The simplest example of association–dissociation between homologous subunits is the monomer–dimer equilibrium. In section 11 below we analyse some effects of this type of mechanism on transients to a stable steady state. The existence of associations between different enzymes (heterologous associations) may generate rate laws that depend on more than one enzyme concentration, showing departures from eqn. (4). Some consequences of homologous and heterologous associations on the control properties of steady-state metabolic concentrations and fluxes have recently been addressed (Kacser *et al.*, 1990; Sauro & Kacser, 1990).

The time courses of some enzymic reactions show 'lags' or 'bursts' under normal assay conditions (see, e.g., Neet & Ainslie, 1980). This phenomenon is associated with the existence of slow conformational transitions in the enzyme mechanism. In these cases the steady-state approximation is not valid, and therefore it is not possible to express the behaviour in time by eqns. (3). The existence of slow conformational changes may have major effects on the control of the time course of a variable, while having no effect on the control properties of the steady state of the system.

Some concentrations of metabolites within a system appear to be linked by conservation constraints (e.g. $[NAD^+]+[NADH]$ = constant). If the total concentrations of enzymes are negligible with respect to the concentrations of conserved metabolites to which they bind, the steady-state approximation is valid. In this frequently considered situation, as there is no significant sequestration of the conserved metabolites by the enzymes, when the CCO is applied eqn. (4) is also valid. Even if the total concentrations of the enzymes are of the same order as the conserved metabolite concentrations, the steady-state assumption may still be satisfied, provided that those concentrations are much smaller than the Michaelis constant (see Segel, 1988). Owing to the low 'affinity' (large Michaelis constant) between enzyme and metabolite, the fraction of the metabolite in complexed form is still small. If, however, the total concentrations of the enzymes and the metabolites are of the same order, but greater than the Michaelis constant, the validity of the steady-state assumption is no longer ensured. Furthermore, in this condition there is considerable sequestration of the conserved metabolites, and we may expect significant deviations in the system variables when the CCO is applied (see Fell & Sauro, 1990).

The enzyme mechanisms mentioned above may be responsible for the appearance of departures from the quantitative relationships derived in sections 5 and 6. Furthermore, they may be the cause of a qualitative change in the dynamics of the system if a 'bifurcation point' is reached when the CCO is applied. Such situations, for example, may transform a sustained oscillation into a stable steady state, or vice versa.

10. QUANTIFICATION OF THE DEVIATIONS

The D-plots and R-plots may be used to test the existence of deviations from the predicted relationships (eqns. 21–24). Such a case is illustrated in Figs. 3(*a*) and 3(*b*). Here we introduce additional plots to assess the quantitative importance of the deviations. These plots constitute a phenomenological description of the deviations. Furthermore, as we show below, they may be useful in the search of the origin of the deviations.

In section 7 we discussed two properties of metabolic variables: (*a*) if Y is a J-type variable (time-dependent or time-invariant), then the values of the variable divided by α, Y/α, behave like an S-type variable (time-dependent or time-invariant respectively); (*b*) if $Y(t)$ is a time-dependent variable (S-type or J-type), then the values of the variable for the same αt and different α, $Y_\alpha(t)$, behave like a time-invariant variable (of the same type). These properties allow us to transform the values of any of the variables described in section 7 into the values of an S-type time-invariant variable. If we want, for example, to compare the deviations of an S-type variable with those of a J-type variable, or to compare the deviations of a time-dependent variable corresponding to different time points, such transformations should be applied. The plots, which we introduce in this section, are defined for S-type time-invariant variables. However, they may also be used in the analysis of other types of variables, with application of the appropriate transformations described above.

To quantify deviations, the data from a CCO experiment may be plotted in, at least, two different ways. In what follows we define and discuss two plots, which we call 'Reference-Point Sensitivity co-ordinate-control plot' (RPS-plot) and 'Point-to-Point Sensitivity co-ordinate-control plot' (PPS-plot).

A. Reference-Point Sensitivity co-ordinate-control plot (RPS-plot)

The RPS-plot is established to characterize the changes of a variable with respect to a unique reference point (point corresponding to $\alpha = 1$). We define a deviation function d_r (for an S-type time-invariant variable) as $d_r = [(S_\alpha - S_r)/S_r]/(\alpha - 1)$. A plot of d_r against α represents the relative change in the value of the variable, with respect to the reference value (S_r), per relative change in the enzyme concentrations, $\alpha - 1$ (see eqn. 2), when the CCO is applied. The ordinate values in this plot would constitute a measure of the quantitative importance of the deviations for different α changes. The sign of the ordinate values is positive or negative if the change in the variable is in the same or opposite direction to the change in the enzyme concentrations respectively. In the absence of deviations the ordinate values d_r are equal to zero for all α.

B. Point-to-Point Sensitivity co-ordinate-control plot (PPS-plot)

In the previous (RPS) plot we used a unique reference point. Alternatively, it is possible to establish a plot where each set of enzyme concentrations serves successively as the reference point.

Let E_{n-1}, E_n and E_{n+1} ($E_{n-1} < E_n < E_{n+1}$) be three consecutive values of the concentration of any one enzyme, and S_{n-1}, S_n and S_{n+1} the corresponding values of an S-type time-invariant variable, resulting from the application of the CCO. The point E_n is momentarily considered as the reference point. The relative change in the variable per relative change in the enzyme concentration from E_n to E_{n+1} is: $d_{+1} = [(S_{n+1} - S_n)/S_n]/[(E_{n+1} - E_n)/E_n]$. Similarly, the relative change in the variable per relative change in the enzyme concentration from E_n to E_{n-1} is: $d_{-1} = [(S_{n-1} - S_n)/S_n]/[(E_{n-1} - E_n)/E_n]$. If the increase and decrease of the enzyme concentration from the reference point are equal (i.e. $E_{n+1} - E_n = E_n - E_{n-1}$), then the relative change in the variable per relative change in the enzyme concentration at the reference point may be estimated by the simple arithmetic mean: $d_n = (d_{+1} + d_{-1})/2$. From p experimental points, $p-2$ values of d_n may be calculated (d_2 to d_{p-1}).

We define the PPS-plot as d_n against E_n. The ordinates in this plot may be considered as an estimate of the deviation in the variable corresponding to each E_n when the CCO is applied. In the absence of deviations the ordinates are equal to zero. The signs of the ordinates are positive or negative if the change in the variable is in the same or the opposite direction to the change in the enzyme concentrations respectively.

It should be noted that if the experimental data are given as S_α against α the relative changes in the enzyme concentrations may be calculated directly from the values of α: $(E_{n+1} - E_n)/E_n = (\alpha_{n+1} - \alpha_n)/\alpha_n$ and $(E_{n-1} - E_n)/E_n = (\alpha_{n-1} - \alpha_n)/\alpha_n$ (see eqn. 2). In addition, the value of d_n may be plotted against α_n.

The arithmetic mean used above to calculate d_n may not be a good estimation when $E_{n+1} - E_n \neq E_n - E_{n-1}$. In this case we propose to use $d_n = [(E_{n+1} - E_n)d_{-1} + (E_n - E_{n-1})d_{+1}]/(E_{n+1} - E_{n-1})$. This weighted mean is equivalent to obtaining the value of d_n by linear interpolation between d_{+1} and d_{-1}. Here the enzyme concentrations may also be substituted by the corresponding α values without changing the results.

There is a link between the values of the ordinates in a PPS-plot and the summation relationships of control analysis. This is given in the Appendix. The construction of these plots is illustrated in section 11.

11. EXAMPLE

Here we show, by way of simulation, how the proposed plots can be used to test and quantify deviations. Although the example chosen is of a monomer–dimer equilibrium (eqn. 4 is violated), the same treatment can be applied to any of the deviations discussed in the preceding section.

We consider one metabolic pathway, whose structure is represented in Scheme 1. The first step is catalysed by an enzyme that presents a monomer–dimer equilibrium. X_0 and X_1 are the constant source and sink concentrations respectively. S is the only metabolite whose concentration is free to alter. The rate for the first step is:

$$v_1 = a_m \cdot M + 2a_d \cdot D \qquad (25)$$

where a_m and a_d are the specific activities of the monomer and dimer subunits respectively. The total concentrations of monomer and dimer, M and D, appearing in eqn. (25), are given by $M = [-1+(1+8K_{app.}E_1)^{\frac{1}{2}}]/(4K_{app.})$ and $D = K_{app.}M^2$. E_1 is the total enzyme concentration expressed in monomer units ($E_1 = M+2D$). $K_{app.}$ (the apparent equilibrium constant), a_m and a_d depend on the concentration, X_0, of the substrate X_0, and are independent of E_1. It should be noted that if $a_m = a_d$ then v_1 is proportional to E_1, and eqn. (4) is fulfilled. Here we consider situations where this is not the case.

The second step in Scheme 1 is catalysed by an irreversible Michaelis–Menten enzyme:

$$v_2 = k_{cat.}E_2\frac{S}{K_m+S}, \qquad (26)$$

E_2 is the total enzyme concentration and $k_{cat.}$ and K_m are constants.

The time course of the metabolite concentration is obtained solving the differential equation:

$$\frac{dS}{dt} = v_1 - v_2 \qquad (27)$$

$$X_0 \xrightarrow{v_1} S \xrightarrow{v_2} X_1$$

Scheme 1.

where v_1 and v_2 are given in eqns. (25) and (26) respectively. Fig. 3(*a*) shows the reference time course, $\alpha = 1$, obtained for a particular set of reference parameter values (given in the legend to Fig. 3) and the time courses after application of the CCO using values of α different from 1. The corresponding curves (not shown) for the flux carried by the second step (flux 2) are obtained by substituting the instantaneous values of the metabolite concentration into eqn. (26).

The R-plots corresponding to Fig. 3(*a*) are shown in Fig. 3(*b*). The five curves in each R-plot do not coincide, and this fact is a positive test for the existence of deviations from the predicted relationship (eqns. 23). Similar results are obtained in the R-plot for flux 2 (not shown), which reveals significant deviations from eqn. (24). We here characterize and quantify these deviations at two different points of time, namely $\alpha t = 1$ and the steady state. The values $S_\alpha^{(1)}$, appearing in Table 1, are the ordinates corresponding to the abscissa $\alpha t = 1$ in the plot of Fig. 3(*b*). The values $J_\alpha^{(1)}$ are calculated from the ordinates $(J_\alpha^{(1)}/\alpha)$ corresponding to the abscissa $\alpha t = 1$ of the R-plot corresponding to flux 2 (not shown). The steady-state values (S_α^{ss} and J_α^{ss}) are the constant values attained after a 'long enough' time. From the steady-state values another time-invariant variable, namely the transition time of the system, can be calculated (Easterby, 1981, 1986): $\tau = S^{ss}/J^{ss}$. Table 1 shows how this value changes with α.

The PPS-plot, calculated from the data of Table 1, appears in Fig. 4. Here the deviations are different, for the different variables (concentration of S, flux 2 and τ) and αt ($\alpha t = 1$ and steady state, for time-dependent variables). Because of the values chosen for the parameters, the deviations are positive. In the case of the metabolite concentrations (S-type variables) positive deviations mean that, when the CCO is applied, the variable moves in the same direction as the enzyme concentrations. For the fluxes and the reciprocal of τ (J-type variables) positive deviations indicate that the change in the variable is greater than the proportional increase expected when the CCO is applied with α greater than unity. It is important to note that the deviation for S^{ss} increases with α, whereas the deviations for the other metabolite concentration and fluxes decrease. These properties of the PPS-plot

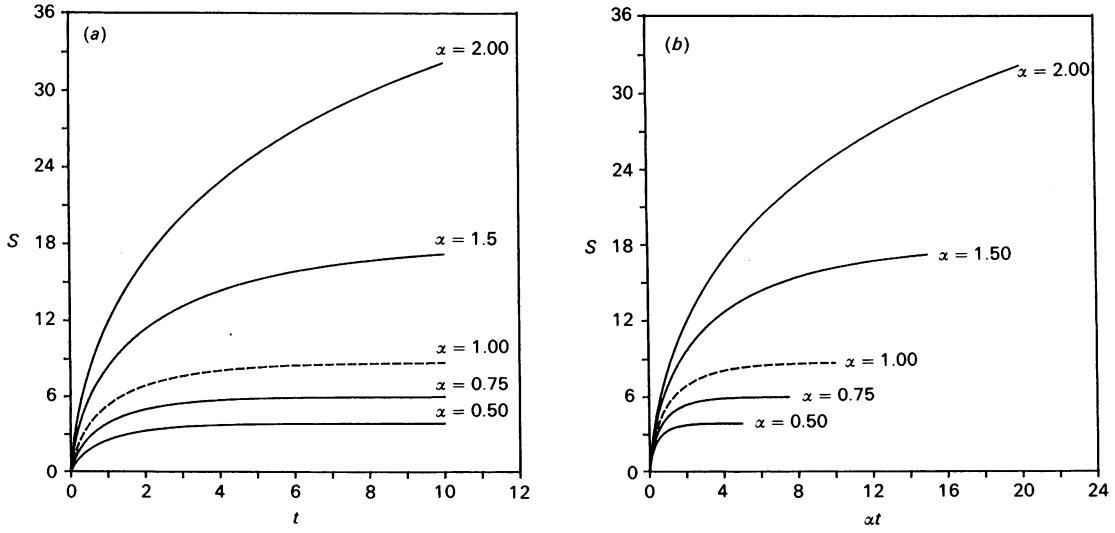

Fig. 3. Example where assumption eqn (4) is not valid, namely monomer–dimer equilibrium: (*a*) concentration of S versus time and (*b*) R-plot

In the example considered in section 11 the reference parameter values used to generate the reference time course ($\alpha = 1$, broken line) are $a_m = 1$, $a_d = 10$, $K_{app.} = 0.1$, $K_A = 3$, $E_1 = 4$ and $V_A = 22$ and the concentration of S at the initial time zero ($S^{in.}$) is zero. The same values for a_m, a_d, $K_{app.}$, K_A and $S^{in.}$ are used to calculate the curves for $\alpha = 0.5$, 0.75, 1.5 and 2.0, and the values of E_1 and V_A are multiplied by the corresponding α (see eqn. 2). The time courses for different α are given in (*a*). In (*b*) the same concentrations of S are plotted against α multiplied by time. The numerical simulations were carried out by using the program SCAMP (Sauro, 1986).

Table 1. Concentration of S and flux 2 (J) versus α

The concentration appearing in this Table may be obtained from Figs. 3(*a*) and 3(*b*). The plots for the fluxes are not shown. The values given correspond to the steady state, ss, and $\alpha t = 1$, (1). τ_α is equal to $S_\alpha^{ss}/J_\alpha^{ss}$.

α	S_α^{ss}	J_α^{ss}	$S_\alpha^{(1)}$	$J_\alpha^{(1)}$	τ_α
0.50	3.90	6.22	3.31	5.77	0.63
0.75	6.02	11.01	4.37	9.78	0.55
1.00	8.76	16.39	5.29	14.04	0.53
1.50	18.12	28.31	6.79	22.88	0.64
2.00	45.77	41.29	7.94	31.94	1.11

constitute a quantitative phenomenological description of the deviations.

In what follows, we analyse how the properties of the component rates (v_1 and v_2) affect the resulting behaviour of the variables when the CCO is applied. This analysis is based on infinitesimal changes ($\alpha \approx 1$) as used in control analysis. It provides us with an interpretation of the deviations appearing in Fig. 4. It can be shown that, in the simple example under consideration, the ordinates of the PPS-plot for the metabolite concentration may be estimated by:

$$D_S(t) = (\pi^{v_1}_{E_1} - 1)C^S_{v_1} \qquad (28)$$

Here, $\pi^{v_1}_{E_1} = (E_1/v_1)(\partial v_1/\partial E_1)$. This π elasticity is equal to unity when the rate v_1 is proportional to the enzyme concentration E_1. $C^S_{v_1} = (v_1/S)/(\partial S/\partial v_1)$ is the Control Coefficient. In general, this Control Coefficient is time-dependent, though in the limit it represents the usual steady-state value (see the Appendix and Kacser *et al.*, 1990). The analogous equation for flux 2 is:

$$D_{J_2}(t) = (\pi^{v_1}_{E_1} - 1)C^{J_2}_{v_1} \qquad (29)$$

It is important to note that eqns. (28) and (29) are valid in this particular example, because $\pi^{v_1}_{E_1}$ is independent of time (in a more general case they must be substituted by expressions that involve integrals). Therefore for this particular example the signs and magnitudes of the deviations in a PPS-plot depend on the product of two factors: (*a*) the sign and magnitude of the deviation of the rate $(\pi^{v_1}_{E_1} - 1)$ and (*b*) the sign and magnitude of the effect that a change in the rate has on the variable ($C^S_{v_1}$ or $C^{J_2}_{v_1}$). The values of these quantities are given in Table 2. In the case studied both factors (*a* and *b*) are positive, which results in a positive deviation in the PPS-plot. The deviation of the rate $(\pi^{v_1}_{E_1} - 1)$ decreases with α. $C^{J_2}_{v_1}$ ($\alpha t = 1$) shows the same tendency, whereas $C^{J_2}_{v_1}$ (steady state) is constant and equal to 1 (first step is irreversible) and $C^S_{v_1}$ ($\alpha t = 1$) increases slightly with α. This dependence on α explains the decreasing curves exhibited by the three variables under consideration (Fig. 4). On the other hand, $C^S_{v_1}$ (steady state) increases with α in such a way that the product $C^S_{v_1}$ $(\pi^{v_1}_{E_1} - 1)$ increases too, being the cause of the increase in the deviation with α in the PPS-plot. It should be pointed out that the increase in $C^S_{v_1}$ is due to an increase in the saturation of the second enzyme with α. However, as S is built up from zero, the saturation effect is not important at the early stages of the time course ($\alpha t = 1$).

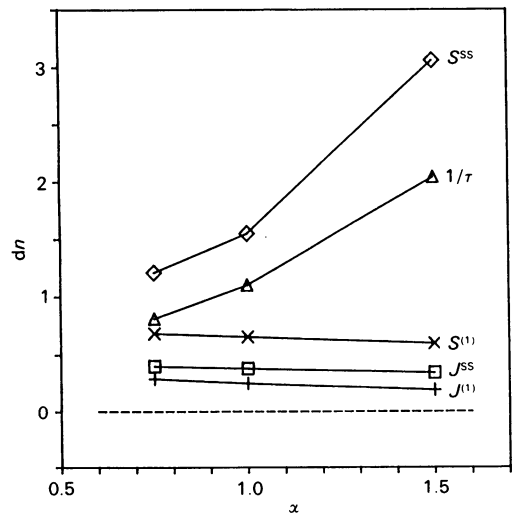

Fig. 4. PPS-plot, d_n against α, in a case where assumption eqn. (4) is not valid: behaviour of time-invariant and time-dependent variables

These plots are constructed by using the data from Table 1. The ordinates corresponding to d_n, S^{ss}_α (◇), $S^{(1)}_\alpha$ (×), J^{ss}_α (□), $J^{(1)}_\alpha$ (+) and $1/\tau_\alpha$ (△) are calculated as described in section 10. The broken line indicates the plot of all variables expected in the absence of deviations.

Table 2. Control Coefficients and deviations

The values of the parameters used to generate the coefficients appearing in this Table are given in Fig. 3. Deviations D_S and D_J were obtained by using eqns. (28) and (29). They are evaluated at two different points: $\alpha t = 1$ and ss (steady state). The values of d_n were obtained from the PPS-plot of Fig. 4. The numerical simulations were carried out by using the program SCAMP (Sauro, 1986).

	α	$C^S_{v_1}$	$C^J_{v_1}$	$\pi^{v_1}_{E_1} - 1$	D_S	d^S_n	D_J	d^J_n
ss	0.75	3.01	1.00	0.39	1.19	1.21	0.39	0.40
	1.00	3.92	1.00	0.37	1.45	1.55	0.37	0.38
	1.50	7.04	1.00	0.33	2.30	3.06	0.33	0.34
$\alpha t = 1$	0.75	1.73	0.70	0.39	0.68	0.68	0.28	0.29
	1.00	1.75	0.63	0.37	0.65	0.65	0.23	0.25
	1.50	1.76	0.54	0.33	0.58	0.59	0.18	0.19

It is important to note that, even though the values of α used to construct the PPS-plot are relatively large, the deviations calculated with eqns. (28) and (29), which are based on infinitesimal changes are in reasonable agreement (see Table 2) with the values of the ordinates, d_n, in Fig. 4, although it is recognized that this need not generally be the case.

Eqns. (28) and (29) illustrate that the existence of strong deviations from proportionality between rate and enzyme concentration (e.g. $\pi^{v_1}_{E_1} \gg 1$) may be irrelevant to the behaviour of a metabolic variable if the magnitude of the Control Coefficient ($C^Y_{v_1}$) is small. However, in other cases the deviation from proportionality in the rate equation may be greatly amplified if the variable shows a high value of the Control Coefficient (e.g. S^{ss} in the situation shown above).

12. DISCUSSION

The ideal CCO consists in the change of all the enzyme concentrations by the same factor, without any alteration in the other parameters of the experiment. [Exceptions are time-invariant input fluxes and time-dependent input metabolite concentrations and fluxes (see sections 4 and 5).] It now remains to discuss how far this operation can be applied to experimental systems. As always, there are special problems that will be encountered in particular applications.

The CCO may be applied to reconstituted systems. These

systems are built up by the use of component molecules that had been previously purified. They are, of course, much more simple than the biological systems that they are intended to mimic. However, they may give insight concerning, for example, the main components and conditions needed to obtain a certain behaviour [see, e.g., Eschrich *et al.* (1980), oscillations; Torres *et al.* (1989), transition time; Salerno *et al.* (1982), transition time]. The composition of a reconstituted system is known and under the control of the experimentalist. Therefore the CCO may, in principle, be applied to reconstituted systems in a simple way. These seem to be the most immediate experimental applications.

In the case of a biological extract, many aspects of the composition are probably not known, which makes it more difficult to apply the CCO. One way to approach this goal might be to take a fraction of the extract and make a complete enzyme inactivation (e.g. denaturation by heating or proteolytic enzymes). By mixing the original extract and the one subjected to inactivation in different proportions, we might obtain dilutions of the active enzyme concentrations without altering the concentrations of the other components of the system. In the ideal conditions the inactivator and the products of inactivation must not react with non-enzymic components of the system. It is evident that the agent used to inactivate the enzymes should be totally removed before the mixing is done. If there is considerable enzyme inactivation during the experiments (spontaneous or induced by unremoved inactivator), the total enzyme concentrations may not be treated as parameters and will constitute a source of deviations. An alternative method consists of successive dilutions of the extract, which would decrease all enzyme concentrations by the same factor. It is, however, necessary to supplement with all the metabolites that are not generated in the system in order to maintain the original concentrations. Such an attempt was made by Das & Busse (1985) in studying glycolytic oscillations in yeast extracts. Although the $[NAD^+]+[NADH]$ and the $[ATP]+[ADP]+[AMP]$ were maintained constant, other cofactors may have been altered by the dilutions. The PPS-plot for the period, which can be constructed with the data obtained from the above publication, shows both positive and negative deviations. This suggests a change of sign of the Control Coefficient, but, in view of the experimental difficulties referred to above, this interpretation may be questionable. If it is desired to extrapolate from experiments on biological extracts to the situation *in vivo*, it is important to note that in the preparation of the extract a dilution takes place. In so doing the quantitative importance of the deviations may be modified.

The application of the CCO to a system *in vivo* appears to be difficult. One might think that the use of, for example, haploid, diploid and tetraploid yeast cells could be a way to achieve this goal. However, in these series the volume increases proportionally to the gene ploidy, leaving most of the enzyme concentrations approximately unchanged (Mortimer, 1958; Ciferri *et al.*, 1969). On the other hand, some enzyme concentrations (e.g. enzymes bound to membranes) may suffer significant changes (Hilger, 1973). This situation is therefore far from what we define as CCO.

A method where enzyme concentrations can be manipulated *in vivo* consists in using conditions when co-ordinate repressions/inductions of pathways occur. These are well known in both fungal and bacterial micro-organisms. By definition the CCO requires the concentrations of all enzymes in the system to be simultaneously altered, and this is certainly not the case in the above systems. Nevertheless, such studies may approach the requirements of a CCO if the system outside the pathway does not interact significantly with it when such repressions/inductions are effected. It is an almost universal observation that single null mutants in one pathway do not impose double (or multiple) requirements on other pathways. This argues against important interactions between pathways. The absence of such interactions, however, will have to be established rigorously or the system will have to be manipulated to eliminate them. An approach to this has been achieved in studying co-ordinate de-repression in the arginine pathway of *Neurospora crassa* (Stuart *et al.*, 1986). Introduction of a regulatory mutant (*cpc-1*) decreases the concentrations of the enzymes by about 3-fold compared with their 'basal' (reference) concentrations in the wild-type. The effects on the flux to arginine of this substantial factorial change, however, are virtually buffered by a strong negative feedback inhibiting an early enzyme of the pathway. When grown in minimal medium, a comparison of the two strains shows only a 16% decrease in the flux in the mutant. This feedback effect can, however, be abolished by growth on citrulline-supplemented medium, which effectively 'shortens' the pathway to the last three steps. When this is done, it is found that the 3-fold decrease in enzyme concentrations results in a 3-fold decrease in flux. In this instance, therefore, no evidence of deviations due to the last three enzymes is observed.

The non-existence of deviations, as a result of a CCO experiment, is informative by itself. This fact indicates that either the assumptions (eqns. 3 and 4) are fulfilled or their violation is unimportant concerning the behaviour of the variables. On the other hand, the discovery of deviations strongly suggests that the properties of one or more components of the network do not coincide with the assumptions made. Furthermore, a positive test for deviations shows that these features of the components have a significant effect on the behaviour of the variables when the enzyme concentrations are changed. The experimental design for performance of the CCO does not necessarily rely on a detailed knowledge of the structure of the metabolic system. However, if we want to have an interpretation of the deviations, the existing profuse amount of information concerning the structure of metabolic systems and the kinetic properties of its component reactions may be useful. This information (e.g. non-proportionality between a rate, v_i, and an enzyme concentration, E_i) may suggest candidates for the 'cause' of the deviations in a variable of the system (Y). To test the candidate, the values of the Control and Elasticity Coefficients (e.g. $C^Y_{E_i}$ and $(\pi^{v_i}_{E_i}-1)$ should be experimentally obtained, in the same conditions used when the CCO was applied (see section 11). We conclude that the CCO and co-ordinate-control plots may be used as a first approach to study the control properties of time-dependent metabolic systems. They constitute a possible way to obtain relevant information and may guide the design of later experiments, leading to a deeper understanding of how metabolic networks work.

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APPENDIX

Relationship of the CCO to control analysis: summation relationships

(*a*) The assumptions of eqns. (3) and (4) of the main paper are valid

Let us consider a time-invariant variable Y. A small relative change in Y, dY/Y, originated by simultaneous small relative changes in all the enzyme concentrations, $dE_j/E_j(=\alpha-1)$, can be written as the sum of the contributions of each individual enzyme:

$$\frac{dY}{Y}=\sum_{j=1}^{m} C^{Y}_{E_j}\cdot\frac{dE_j}{E_j} \qquad \text{(A1)}$$

where $C^{Y}_{E_j}$ is the Control Coefficient of the variable Y, by the enzyme concentration E_j, and it is defined as follows:

$$C^{Y}_{E_j}\equiv\frac{E_j}{Y}\cdot\frac{\partial Y}{\partial E_j} \qquad \text{(A2)}$$

When the changes in E_j correspond to a CCO (see eqn. 2) $dE_j/E_j=\alpha-1$ (for $j=1,\ldots,m$). If the assumptions introduced in eqns. (3) and (4) of the main paper are valid, the relative change in the variable, dY/Y, is zero in the case of an S-type time-invariant variable, S, and $\alpha-1$ for a J-type time-invariant variable, J (see eqns. 21 and 22 of the main paper). Introducing these results into eqn. (A1), we obtain:

$$\sum_k C^{S}_{E_k}=0 \qquad \text{(A3)}$$

$$\sum_k C^{J}_{E_k}-1=0 \qquad \text{(A4)}$$

Eqns. (A3) and (A4) are the summation relationships for S-type and J-type time-invariant variables respectively. Examples of these variables are given in part A of section 7 of the main paper. Particular cases of eqns. (A3) and (A4) are the summation relationships for the Control Coefficients of the steady-state metabolite concentrations and fluxes (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). The reciprocal of a time-invariant variable with dimension of time, $1/T$, fulfils eqn. (A4). Note that $C^{1/T}_{E_k}=-C^{T}_{E_k}$ and therefore, for these variables:

$$\sum_k C^{T}_{E_k}=-1$$

This general relationship was previously obtained for particular definitions of transition time (see Heinrich & Rapoport, 1975; Torres *et al.*, 1989; Meléndez-Hevia *et al.*, 1990), but is general for any variable that obeys the transformation eqn. (10) of the main paper.

The summation relationships for the Control Coefficients of S-type and J-type time-dependent variables can be written as follows:

$$\sum_k C^{S}_{E_k}-T^{S}=0 \qquad \text{(A5)}$$

$$\sum_k C^{J}_{E_k}-1-T^{J}=0 \qquad \text{(A6)}$$

The 'Time Coefficients' T^S and T^J are defined by $T^Y=(t/Y)/(\partial Y/\partial t)$, where Y stands for S or J (see Acerenza *et al.*, 1989).

(*b*) Deviations from the assumptions of eqns. (3) and (4) of the main paper

Eqns. (A3)–(A6) are derived by using the assumptions introduced in eqns. (3) and (4) of the main paper. If these assumptions are not fulfilled, the left-hand members of eqns. (A3)–(A6) are not equal to zero. It may be shown that, when the CCO is applied with small changes ($\alpha\approx 1$), those left-hand members are not equal to zero but are approximately equal to a deviation term (D) given by eqns. (A7)–(A10) respectively:

$$D_S=\frac{\left(\frac{S_\alpha-S_r}{S_r}\right)}{\alpha-1} \qquad \text{(A7)}$$

$$D_J=\frac{\left(\frac{(J_\alpha/\alpha)-J_r}{J_r}\right)}{\alpha-1} \qquad \text{(A8)}$$

$$D_S(t)=\frac{\left(\frac{S_\alpha(\alpha t_\alpha=t)-S_r(t)}{S_r(t)}\right)}{\alpha-1} \qquad \text{(A9)}$$

$$D_J(t)=\frac{\left(\frac{[J_\alpha(\alpha t_\alpha=t)/\alpha]-J_r(t)}{J_r(t)}\right)}{\alpha-1} \qquad \text{(A10)}$$

It is important to note that these D values are the better approximations to the left-hand members of eqns. (A3)–(A6) the closer α tends to 1.

The deviations given in eqns. (A7)–(A10) are those plotted against α in a PPS-plot (see section 10 of the main paper). The only difference is that in the PPS-plot we use the mean between positive and negative $\alpha-1$ values to compensate (partially) the

error introduced by the use of relatively large changes. It is easy to show that all D values are zero when the assumptions eqns. (3) and (4) of the main paper apply.

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