Determination of control coefficients in intact metabolic systems

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The control structure of a metabolic system can in principle be determined without the need for purification of the component enzymes and study of their kinetic properties, provided that their activities can be perturbed by amounts sufficient to produce measurable changes in the steady-state variables, i.e. the fluxes through the system and the concentrations of the intermediates. Each perturbation is characterized in terms of the co-response coefficients of all pairs of variables, i.e. the slopes of the lines produced when the logarithm of one variable is plotted against the logarithm of another, both varying in response to the same perturbation. If all the co-response coefficients are assembled into a matrix, the inverse of this matrix can be transformed into

a matrix containing all the component elasticities, which can be inverted to provide the complete matrix of control coefficients. In a simple three-enzyme pathway studied, the analysis proves not to require unrealistically high accuracy in the original co-response measurements: even with errors with standard deviation $\pm 5.77^\circ$ in the angles to the horizontal of the lines in the co-response plots (equivalent at best to errors of $\pm 20\%$ in the corresponding co-response coefficients), the final control coefficient matrix may be adequate for assessing the control structure of the system. Examination of literature data from studies of mitochondrial respiration and of gluconeogenesis indicates that considerably higher precision than this is achievable.

INTRODUCTION

Metabolic control analysis (Kacser and Burns, 1973; Heinrich and Rapoport, 1974) allows the control structure of a metabolic system to be expressed quantitatively [see Fell (1992) or Cornish-Bowden (1994) for current reviews]. Various methods have been used for determining the control coefficients of metabolic systems, all of them (apart from the double-modulation method, discussed in the next paragraph) requiring some knowledge of the kinetic properties of the component enzymes. Although genetic methods, such as those used in the studies of arginine synthesis in *Neurospora crassa* (Flint et al., 1981) and of tryptophan synthesis in *Saccharomyces cerevisiae* (Niederberger et al., 1992), depend primarily on measurements on the intact system, they still require knowledge of the magnitudes of the changes in enzyme activity; inhibitor methods, such as those developed by Groen et al. (1982a,b) to study gluconeogenesis and oxidative phosphorylation, and also used by Salter et al. (1986) to study aromatic amino acid metabolism, require even more kinetic information.

We recently showed that, at least in principle, the control structure of a system can be measured without the need for the component enzymes to be purified or for the changes in their activities to be known, though it remains necessary to establish that each perturbation acts on a unique identified enzyme (Hofmeyr et al., 1993). It is sufficient to perturb each enzyme in turn (by an unknown amount) and to measure the simultaneous effects of the perturbation on the system variables. Our approach has some similarities to the double-modulation method suggested by Kacser and Burns (1979), which allows certain elasticities to be measured by comparing the responses of two variables to two independent perturbations, of either enzyme activities or concentrations of pool metabolites.

As our proposal arose incidentally during an analysis done for a different purpose, the development of a quantitative theory of regulation (Hofmeyr and Cornish-Bowden, 1991), we did not discuss it thoroughly or determine how much precision in the measurements would be needed for it to be of practical use. We now describe the calculations in detail for a simple example, and show that the necessary precision is by no means unattainable and has, in fact, been attained in published experiments. The

calculation can be done in n^n different ways, for a pathway of n enzymes, because only a selection of the measured responses are actually used in each. The differences in results obtained by making different choices from the n^n possibilities prove to be real but slight.

TERMINOLOGY

The control coefficient of an enzyme E_i for any steady-state variable y is defined (Burns et al., 1985) as the ratio of the scaled partial derivatives of the variable and of the rate v_i of the isolated enzyme with respect to any parameter p that affects the activity of E_i :

$$C_i^y = \frac{\partial \ln y / \partial p}{\partial \ln v_i / \partial p} \quad (1)$$

The kinetic properties of the individual enzymes are expressed in terms of their elasticities with respect to all metabolites that interact with them, so that, for example, the elasticity $\epsilon_{s_j}^{v_i}$ of E_i with respect to the concentration s_j of a metabolite S_j is defined as follows:

$$\epsilon_{s_j}^{v_i} = \frac{\partial \ln v_i}{\partial \ln s_j} \quad (2)$$

These are the fundamental quantities used in control analysis. For the present paper we require a third coefficient, the co-response coefficient $E_i O_{v_2}^{v_1}$ (Hofmeyr et al., 1993), which measures the tendency of two variables y_1 and y_2 (two fluxes, a flux and a concentration, or two concentrations) to vary concomitantly when an enzyme E_i is perturbed:

$$E_i O_{v_2}^{v_1} = C_i^{v_1} / C_i^{v_2} \quad (3)$$

The essential experimental feature of the co-response coefficient is that it can be measured in an intact system without knowing the magnitude of the perturbation in the activity of E_i . Although the individual control coefficients that appear in its definition are defined with reference to known changes in this activity, these changes cancel from eqn. (3) provided that the perturbations in y_1 and y_2 are measured under the same conditions, i.e. in response to the same perturbation.

Figure 1 Pathway of three enzymes

The broken lines represent potential feedback or feedforward interactions that were absent from the model as simulated (and are marked with crosses to indicate this) but which were not assumed to be absent when the co-response coefficients were transformed arithmetically into elasticities and control coefficients. The reaction arrows indicate the forward direction of the flux through the pathway; they do not imply that any reaction is irreversible. The rate equations were as follows: $v_1 = e_1(10x_0 - 3s_1)/(2 + x_0 + 2s_1)$, $v_2 = e_2(7s_1 - s_2)/(3 + 2s_1 + s_2)$, $v_3 = 11e_3s_2/(1 + 2s_2)$; and the concentrations of X_0 and X_3 were fixed at $x_0 = 2.5$, $x_3 = 0$. For enzyme concentrations of $e_1 = e_2 = e_3 = 1$, these values generate a steady state in which $J = v_1 = v_2 = v_3 = 2$ (exactly), and $s_1 = 2.286$, $s_2 = 0.2857$.

Table 1 Protocol for complete analysis of the model of Figure 1

1. Perturb the activity of E_1 either up or down (preferably both) by means of specific inhibitors or activators or by genetic manipulation, and measure the effects of the perturbation on the flux J and the intermediate concentrations s_1 and s_2 .
2. a. Plot $\log s_1$ against $\log J$ and measure the slope at the point corresponding to the initial steady state to obtain $\epsilon_{10}^{s_1}$;
b. Plot $\log s_2$ against $\log J$ and measure the slope at the point corresponding to the initial steady state to obtain $\epsilon_{10}^{s_2}$.
3. Repeat steps 1 and 2 for a similar perturbation of E_2 , to get $\epsilon_{20}^{s_1}$ and $\epsilon_{20}^{s_2}$.
4. Repeat steps 1 and 2 for a similar perturbation of E_3 , to get $\epsilon_{30}^{s_1}$ and $\epsilon_{30}^{s_2}$.
5. Use these values to construct the matrix
$$\begin{bmatrix} 1 & -\epsilon_{10}^{s_1} & -\epsilon_{10}^{s_2} \\ 1 & -\epsilon_{20}^{s_1} & -\epsilon_{20}^{s_2} \\ 1 & -\epsilon_{30}^{s_1} & -\epsilon_{30}^{s_2} \end{bmatrix}$$
.
6. Proceed through the steps shown by eqns. (7–10) of the text, i.e.
 - a. Invert the co-response coefficient matrix.
 - b. Divide all elements on both sides by the top-row elements.
 - c. Invert the resulting elasticity matrix to get the control coefficient matrix.

COMPUTATION

The behaviour of the three-enzyme pathway illustrated in Figure 1 was simulated on an IBM-PC compatible computer with the program MetaModel (Cornish-Bowden and Hofmeyr, 1991).

To simulate experimental error, normally distributed values with specified standard deviations were generated by applying the transformation of Box and Muller (1958) to uniformly distributed pseudo-random numbers generated with a mixture of three linear congruential generators as described by Press et al. (1986).

MEASUREMENT OF CO-RESPONSE COEFFICIENTS

We illustrate the measurement of co-response coefficients by reference to the model of Figure 1, following the protocol set out in Table 1. Each enzyme activity must be perturbed by addition of specific inhibitors or activators, or by genetic manipulation, etc., in turn by amounts sufficient to produce measurable changes in the flux J through the pathway and in the concentrations s_1 and s_2 of the two metabolites.

For each of the three series of perturbations, a double logarithmic plot of each variable against each other variable yields a co-response profile, from which the slope of the tangent at the point corresponding to the original steady state yields the appropriate co-response coefficient. A complete set of co-response profiles is illustrated in Figure 2. These are idealized in

Figure 2 Co-response profiles

The Figure shows the nine co-response profiles for the model of Figure 1. Each curve shows how a particular pair of variables (top left, J and s_1 ; top right, s_2 and s_1 ; bottom left, J and s_2) responds to a perturbation in the activity of one enzyme, as marked with the arrows. Two pairs of curves are superimposed; thus only seven distinct curves are visible in the Figure. As each curve represents a two-dimensional projection of a curve in three dimensions, the panels are laid out in the style of a 'rug-plot' [(Tuft, 1983) or see Cornish-Bowden and Endrenyi (1986) for a different biochemical example] to facilitate finding corresponding points in the three panels: the dotted line indicates this correspondence in the case of the operating point, i.e. the initial steady state at which all the slopes are to be estimated.

Figure 3 Determination of a co-response coefficient

One of the curves from Figure 2 is reproduced on a larger scale. The tangent at the operating point makes an angle of $\epsilon_{20}^{s_1} = -48.5^\circ$ with the $\log J$ axis, and its slope is the value of $\epsilon_{20}^{s_1} = -1.13$ that appears in Table 2.

Table 2 Co-response coefficients for the model of Figure 1

The Table shows the co-response coefficients used for illustrating the calculation. They refer to the steady state of the model of Figure 1 with flux of $J = 2.000$, and intermediate concentrations $s_1 = 2.286$, $s_2 = 0.2857$. Note that for any enzyme E_i and variables y_1 and y_2 , $\epsilon_{iO_{y_1}}^J \equiv 1$ and $\epsilon_{iO_{y_2}}^J \equiv 1/\epsilon_{iO_{y_1}}^J$. Other equalities evident in the Table are diagnostic for particular characteristics of the pathway, as discussed in the text.

E_i	$\epsilon_{iO_J}^J$	$\epsilon_{iO_{s_1}}^J$	$\epsilon_{iO_{s_2}}^J$	$\epsilon_{iO_J}^{s_1}$	$\epsilon_{iO_{s_1}}^{s_1}$	$\epsilon_{iO_{s_2}}^{s_1}$	$\epsilon_{iO_J}^{s_2}$	$\epsilon_{iO_{s_1}}^{s_2}$	$\epsilon_{iO_{s_2}}^{s_2}$
E_1	1.00	0.40	0.64	2.50	1.00	1.58	1.57	0.63	1.00
E_2	1.00	-0.88	0.64	-1.13	1.00	-0.72	1.57	-1.39	1.00
E_3	1.00	-0.88	-0.04	-1.13	1.00	0.04	-27.4	24.2	1.00

the sense that each enzyme activity has been perturbed in both directions from the operating steady state: in practice this will often not be possible, and activities will be either decreased or increased, but not both; such asymmetry in the experimental design may be a source of bias in the estimates of co-response coefficients, but is probably unavoidable. Although the lines are curved, illustrating that co-response coefficients are not constant but depend on the particular steady state considered, the curvature is relatively slight: this means that one can hope to obtain reasonably accurate values of the coefficients from measurements in which the steady-state variables change by conveniently measurable factors. Note also that some of the co-response profiles are superimposable: this is a potentially useful diagnostic feature that we discuss below.

Figure 3 illustrates the calculation of the co-response coefficient for one of the curves from Figure 2. For simplicity, we assume here that the slope can be estimated directly from the graph. If computation is used, it is important to realize that conventional linear and non-linear regression methods treat one variable as dependent and the other(s) as independent, i.e. they treat the variables unsymmetrically, with the result that $\hat{O}_{y_1}^{y_2}$ will not be the same as $1/\hat{O}_{y_2}^{y_1}$ if $\hat{O}_{y_1}^{y_2}$ is calculated treating y_2 as dependent variable and $\hat{O}_{y_2}^{y_1}$ is calculated treating y_1 as dependent variable. This type of asymmetry is not at all desirable, and can lead to bias in the final result. Methods of calculation that correctly treat both y_1 and y_2 as dependent variables are discussed in the Appendix.

For the remainder of this paper we shall not consider explicitly how the co-response coefficients are estimated but will discuss how they can be used once they are available. Table 2 lists the complete set of co-response coefficients for the steady state of the model of Figure 1 for which $x_0 = 2.5$ and $x_3 = 0$.

CONVERSION OF CO-RESPONSE COEFFICIENTS TO ELASTICITIES

The fundamental relationships of control analysis may be expressed as a matrix equation. The general formulation given by various authors (e.g. Westerhoff and Kell, 1987) assumes the following form in the case of Figure 1:

$$\begin{bmatrix} 1 & 1 & 1 \\ \epsilon_{s_1}^{v_1} & \epsilon_{s_1}^{v_2} & \epsilon_{s_1}^{v_3} \\ \epsilon_{s_2}^{v_1} & \epsilon_{s_2}^{v_2} & \epsilon_{s_2}^{v_3} \end{bmatrix} \begin{bmatrix} C_1^J & -C_1^{s_1} & -C_1^{s_2} \\ C_2^J & -C_2^{s_1} & -C_2^{s_2} \\ C_3^J & -C_3^{s_1} & -C_3^{s_2} \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix} \quad (4)$$

We do not need to assume that any of the entries in the elasticity matrix are zero. Although knowledge of the enzymes concerned may suggest, for example, that S_1 has no effect on E_3 , i.e. that $\epsilon_{s_1}^{v_3} = 0$, such zeroes ought to emerge from the analysis without being assumed at the outset.

Post-multiplying the elasticity matrix by a diagonal matrix D^v with diagonal elements $[C_1^J, C_2^J, C_3^J]$, and pre-multiplying the

control coefficient matrix by its inverse, i.e. by a diagonal matrix with diagonal elements $[1/C_1^J, 1/C_2^J, 1/C_3^J]$, one obtains the following relationship (Hofmeyr et al., 1993):

$$\begin{bmatrix} C_1^J & C_2^J & C_3^J \\ \epsilon_{s_1}^{v_1} C_1^J & \epsilon_{s_1}^{v_2} C_2^J & \epsilon_{s_1}^{v_3} C_3^J \\ \epsilon_{s_2}^{v_1} C_1^J & \epsilon_{s_2}^{v_2} C_2^J & \epsilon_{s_2}^{v_3} C_3^J \end{bmatrix} \begin{bmatrix} E_1 O_J^J & -E_1 O_{s_1}^J & -E_1 O_{s_2}^J \\ E_2 O_J^J & -E_2 O_{s_1}^J & -E_2 O_{s_2}^J \\ E_3 O_J^J & -E_3 O_{s_1}^J & -E_3 O_{s_2}^J \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix} \quad (5)$$

This shows that the matrix of products of elasticities and control coefficients is the inverse of the matrix of co-response coefficients:

$$\begin{bmatrix} C_1^J & C_2^J & C_3^J \\ \epsilon_{s_1}^{v_1} C_1^J & \epsilon_{s_1}^{v_2} C_2^J & \epsilon_{s_1}^{v_3} C_3^J \\ \epsilon_{s_2}^{v_1} C_1^J & \epsilon_{s_2}^{v_2} C_2^J & \epsilon_{s_2}^{v_3} C_3^J \end{bmatrix} = \begin{bmatrix} E_1 O_J^J & -E_1 O_{s_1}^J & -E_1 O_{s_2}^J \\ E_2 O_J^J & -E_2 O_{s_1}^J & -E_2 O_{s_2}^J \\ E_3 O_J^J & -E_3 O_{s_1}^J & -E_3 O_{s_2}^J \end{bmatrix}^{-1} \quad (6)$$

Although all of the entries in the first column of the co-response coefficient matrix are identical to 1, they are written here as algebraic symbols to make the relationship to eqn. (4) more transparent. With the numerical values given in Table 2, eqn. (6) becomes:

$$\begin{bmatrix} C_1^J & C_2^J & C_3^J \\ \epsilon_{s_1}^{v_1} C_1^J & \epsilon_{s_1}^{v_2} C_2^J & \epsilon_{s_1}^{v_3} C_3^J \\ \epsilon_{s_2}^{v_1} C_1^J & \epsilon_{s_2}^{v_2} C_2^J & \epsilon_{s_2}^{v_3} C_3^J \end{bmatrix} = \begin{bmatrix} 1 & -2.50 & -1.57 \\ 1 & 1.13 & -1.57 \\ 1 & 1.13 & 27.4 \end{bmatrix}^{-1} = \begin{bmatrix} 0.31129 & 0.63451 & 0.05419 \\ -0.27548 & 0.27548 & 0.00000 \\ 0.00000 & -0.03452 & 0.03452 \end{bmatrix} \quad (7)$$

The top row of the right-hand side of this equation contains all the flux control coefficients, i.e.

$$[C_1^J \ C_2^J \ C_3^J] = [0.31129 \ 0.63451 \ 0.05419] \quad (8)$$

and if these are all that are required the calculation is finished. However, it is easy to obtain the concentration control coefficients as well. To do this, the matrix given in eqn. (7) must first be converted into the elasticity matrix by dividing each element by the value at the top of its column:

$$\begin{bmatrix} 1 & 1 & 1 \\ \epsilon_{s_1}^{v_1} & \epsilon_{s_1}^{v_2} & \epsilon_{s_1}^{v_3} \\ \epsilon_{s_2}^{v_1} & \epsilon_{s_2}^{v_2} & \epsilon_{s_2}^{v_3} \end{bmatrix} = \begin{bmatrix} 1.00000 & 1.00000 & 1.00000 \\ -0.88496 & 0.43416 & 0.00000 \\ 0.00000 & -0.05440 & 0.63702 \end{bmatrix} \quad (9)$$

Note that the two zeroes in this matrix are the result of the arithmetic, and do not arise from any initial assumptions.

Inversion of this matrix then gives the complete control coefficient matrix by virtue of eqn. (4):

$$\begin{aligned} & \begin{bmatrix} C_1^J & -C_1^{s_1} & -C_1^{s_2} \\ C_2^J & -C_2^{s_1} & -C_2^{s_2} \\ C_3^J & -C_3^{s_1} & -C_3^{s_2} \end{bmatrix} \\ &= \begin{bmatrix} 1.00000 & 1.00000 & 1.00000 \\ -0.88496 & 0.43416 & 0.00000 \\ 0.00000 & -0.05440 & 0.63702 \end{bmatrix}^{-1} \\ &= \begin{bmatrix} 0.31129 & -0.77823 & -0.48867 \\ 0.63452 & 0.71700 & -0.99607 \\ 0.05419 & 0.06123 & 1.48475 \end{bmatrix} \quad (10) \end{aligned}$$

A MORE REALISTIC EXAMPLE

As this calculation has been done starting with a co-response coefficient matrix that was essentially free from error, it may seem too unrealistic to be meaningful. We therefore repeat it with one of the sets of data used for constructing Table 3 below, i.e. with data with substantial errors in the co-response coefficient matrix. Comparing the following equation with eqn. (7), it is evident that five of the values differ from the correct ones by errors in the range 5–37%, and the sixth differs from the correct value by a factor of about –10:

$$\begin{aligned} & \begin{bmatrix} C_1^J & C_2^J & C_3^J \\ \epsilon_{s_1}^{v_1} C_1^J & \epsilon_{s_1}^{v_2} C_2^J & \epsilon_{s_1}^{v_3} C_3^J \\ \epsilon_{s_2}^{v_1} C_1^J & \epsilon_{s_2}^{v_2} C_2^J & \epsilon_{s_2}^{v_3} C_3^J \end{bmatrix} = \begin{bmatrix} 1 & -2.63 & -0.99 \\ 1 & 1.04 & -1.70 \\ 1 & 0.96 & -2.60 \end{bmatrix}^{-1} \\ &= \begin{bmatrix} 0.28351 & 0.72230 & -0.00580 \\ -0.27246 & 0.27321 & -0.00075 \\ 0.00008 & 0.00379 & -0.00387 \end{bmatrix} \quad (11) \end{aligned}$$

By the same transformations as before, this yields the following elasticity and control coefficient matrices:

$$\begin{aligned} & \begin{bmatrix} 1 & 1 & 1 \\ \epsilon_{s_1}^{v_1} & \epsilon_{s_1}^{v_2} & \epsilon_{s_1}^{v_3} \\ \epsilon_{s_2}^{v_1} & \epsilon_{s_2}^{v_2} & \epsilon_{s_2}^{v_3} \end{bmatrix} = \begin{bmatrix} 1.00000 & 1.00000 & 1.00000 \\ -0.96102 & 0.37825 & 0.12930 \\ 0.00028 & 0.00525 & 0.66724 \end{bmatrix} \quad (12) \\ & \begin{bmatrix} C_1^J & -C_1^{s_1} & -C_1^{s_2} \\ C_2^J & -C_2^{s_1} & -C_2^{s_2} \\ C_3^J & -C_3^{s_1} & -C_3^{s_2} \end{bmatrix} \\ &= \begin{bmatrix} 0.28351 & -0.74563 & -0.28041 \\ 0.72229 & 0.75123 & -1.22809 \\ -0.00580 & 0.00560 & 1.50849 \end{bmatrix} \quad (13) \end{aligned}$$

This control coefficient matrix gives qualitatively correct information: in all three columns the entries are in the correct order and are similar in magnitude to the correct values shown in eqn. (10). There are likewise no major anomalies in the elasticity matrix, though it is possibly worth mentioning a spurious suggestion of weak feedforward activation of E_3 by S_1 . As will be shown below, this sort of result is typical: even a co-response coefficient matrix containing quite large errors will usually lead to useful information in the final control coefficient matrix.

CHOICE OF ENTRIES IN THE DIAGONAL MATRIX D^J

In the examples we used a diagonal matrix D^J containing flux control coefficients $[C_1^J, C_2^J, C_3^J]$ for transforming eqn. (4) into

eqn. (5). This is only one out of 27 possible choices of diagonal matrix (in general there are n^n possible choices for a pathway of n enzymes), as each of the three superscripts can be any of the three variables J, s_1 or s_2 . Our earlier discussion (Hofmeyr et al., 1993) may have given the impression that each variable had to appear once as superscript, but, as the above examples illustrate, the calculation works satisfactorily without any such restriction, and the particular choice $[C_1^J, C_2^J, C_3^J]$ has the advantage of yielding the flux control coefficients after only one matrix inversion. Nonetheless, if there is significant experimental error in the co-response coefficients, as will always be the case in a real experiment, the 27 choices lead to 27 different results for the final control matrix, and the question then arises as to whether any choices are better than others.

As a co-response coefficient can have any value, either positive or negative, including values near zero and near infinity, it is inappropriate to assume that its errors have either constant

Table 3 Combinations of variables in D^J

The Table shows the standard deviations of the flux control coefficients and elasticities obtained with each of the 27 possible combinations of control coefficients in D^J , the diagonal matrix used for transforming eqn. (3) into eqn. (5), for two levels of error in the co-response coefficients. Each value in the Table was obtained from 5000 simulated sets of co-response coefficients. As discussed in the text, errors of 2.87° and 5.77° in the angle corresponding to a co-response coefficient are equivalent to errors of 10% and 20% respectively in values with true values of 1.00. (More detail of the correspondence is given in Table 4.) The row labelled 'mean' shows standard deviations over the 5000 simulations of the means of the 27 values of each flux control coefficient estimated in each of the 27 possible ways; the row labelled 'mean (e)' shows standard deviations of flux control coefficients calculated by inverting the matrices of elasticities calculated as means of the 27 values of each elasticity estimated in each of the 27 possible ways; the row labelled 'median' shows standard deviations of the medians of the 27 values of each flux control coefficient estimated in each of the 27 possible ways, corrected to satisfy the summation relationship by dividing each median by the sum of the three medians.

Combination	Errors of 2.87°		Errors of 5.77°	
	$\sigma(C^J)$	$\sigma(e)$	$\sigma(C^J)$	$\sigma(e)$
$[C_1^J, C_2^J, C_3^J]$	0.0677	0.0661	0.1590	0.8660 max.
$[C_1^J, C_2^J, C_3^{s_1}]$	0.0613	0.0627 min.	0.1397	0.1357
$[C_1^J, C_2^J, C_3^{s_2}]$	0.0638	0.0758	0.1485	0.1675
$[C_1^J, C_2^{s_1}, C_3^J]$	0.0679	0.0689	0.1649	0.1815
$[C_1^J, C_2^{s_1}, C_3^{s_1}]$	0.0617	0.0658	0.1514	0.1420
$[C_1^J, C_2^{s_1}, C_3^{s_2}]$	0.0642	0.0780	0.1505	0.1671
$[C_1^J, C_2^{s_2}, C_3^J]$	0.0703	0.0778	0.1586	0.2042
$[C_1^J, C_2^{s_2}, C_3^{s_1}]$	0.0644	0.0751	0.1426	0.1663
$[C_1^J, C_2^{s_2}, C_3^{s_2}]$	0.0668	0.0866	0.1506	0.1948
$[C_1^{s_1}, C_2^J, C_3^J]$	0.0675	0.0679	0.1609	0.8639
$[C_1^{s_1}, C_2^J, C_3^{s_1}]$	0.0609 min.	0.0648	0.1392 min.	0.1366
$[C_1^{s_1}, C_2^J, C_3^{s_2}]$	0.0635	0.0776	0.1485	0.1697
$[C_1^{s_1}, C_2^{s_1}, C_3^J]$	0.0676	0.0707	0.1644	0.1710
$[C_1^{s_1}, C_2^{s_1}, C_3^{s_1}]$	0.0614	0.0677	0.1480	0.1427
$[C_1^{s_1}, C_2^{s_1}, C_3^{s_2}]$	0.0639	0.0798	0.1504	0.1692
$[C_1^{s_1}, C_2^{s_2}, C_3^J]$	0.0700	0.0793	0.1601	0.1949
$[C_1^{s_1}, C_2^{s_2}, C_3^{s_1}]$	0.0641	0.0768	0.1424	0.1670
$[C_1^{s_1}, C_2^{s_2}, C_3^{s_2}]$	0.0665	0.0882 max.	0.1510	0.1966
$[C_1^{s_2}, C_2^J, C_3^J]$	0.0681	0.0663	0.1573	0.8638
$[C_1^{s_2}, C_2^J, C_3^{s_1}]$	0.0617	0.0630	0.1396	0.1357
$[C_1^{s_2}, C_2^J, C_3^{s_2}]$	0.0643	0.0761	0.1465	0.1682
$[C_1^{s_2}, C_2^{s_1}, C_3^J]$	0.0683	0.0692	0.1671 max.	0.1704
$[C_1^{s_2}, C_2^{s_1}, C_3^{s_1}]$	0.0621	0.0660	0.1560	0.1417 min.
$[C_1^{s_2}, C_2^{s_1}, C_3^{s_2}]$	0.0646	0.0783	0.1502	0.1675
$[C_1^{s_2}, C_2^{s_2}, C_3^J]$	0.0709 max.	0.0780	0.1575	0.1945
$[C_1^{s_2}, C_2^{s_2}, C_3^{s_1}]$	0.0651	0.0753	0.1429	0.1662
$[C_1^{s_2}, C_2^{s_2}, C_3^{s_2}]$	0.0675	0.0868	0.1496	0.1952
Mean	0.0493		0.1126	
Mean (e)	0.0487		0.1039	
Median	0.0603		0.1423

Figure 4 Results from 100 simulated experiments

The flux control coefficients from 100 simulations done with angular errors of $\pm 2.87^\circ$ in the co-response coefficients are plotted in triangular coordinates. Each apex of the principal triangle represents a value of unity for the control coefficient shown as label, and values of zero for the two others; 97% of the points show $C_2^J > 0.5$, and 100% show $C_2^J > C_1^J > C_3^J$, i.e. they all give a qualitatively correct impression of the distribution of flux control.

variance or constant coefficient of variation. It is better to suppose that each co-response coefficient $E_i O_{y_1}^{y_2}$ corresponds to an angle $E_i \theta_{y_1}^{y_2} = \arctan E_i O_{y_1}^{y_2}$ that is subject to an error with specified statistical properties. For a value of $E_i O_{y_1}^{y_2} = 1.0$ ($E_i \theta_{y_1}^{y_2} = 45^\circ$), errors of $\pm 10\%$ and $\pm 20\%$ give errors of $\pm 2.87^\circ$ (± 0.0501 radian) and $\pm 5.77^\circ$ (± 0.1006 radian) respectively in the corresponding angles. To simulate effects of experimental error on the calculations, therefore, we have converted all of the co-response coefficients into angles, added normally distributed errors with mean zero and standard deviation 2.87° or 5.77° , and converted the resulting angles back into co-response coefficients. At each error level, 5000 sets of data were generated, and each set of data was analysed in each of the 27 possible ways, i.e. the steps from eqn. (4) to eqn. (9) were carried out with each set of data with each of the 27 possible choices of D^J . The results (Table 3) were compared by calculating, for each D^J , the standard deviations of the three flux control coefficients:

$$\text{Standard deviation} = \left[\frac{1}{15000} \sum_{j=1}^3 \sum_{i=1}^3 (C_i^J - C_{i,\text{true}}^J)^2 \right]^{\frac{1}{2}} \quad (14)$$

and likewise for the six elasticities.

The differences between the 27 combinations are small, but repeated simulations generally agree about which choices yield the best and the worst results: for example, the largest value normally corresponds to a D^J that contains C_3^J , whereas the smallest value corresponds to a D^J that contains C_1^J . The objection to C_3^J is probably that it is the nearest to zero of the three flux control coefficients; the merits of C_3^J seem less obvious. The suggestion (Hofmeyr et al., 1993) that one should avoid control coefficients with values close to zero appears to have some validity, although the complete selection may be both less straightforward and less important than it initially appeared.

At the higher error level, rare data sets produce very large errors for some choices of D^J : about 0.1% gave flux control coefficients that differed on average from the true values by more than 1. Provided that the number of simulations is sufficiently

Figure 5 Lack of independence of different choices of D^J

The 27 sets of flux control coefficients with different D^J matrices produced from the same data are plotted in triangular co-ordinates as in Figure 4. The eight points (of which one pair is not resolved) lying on or close to the line labelled 'not O^1 ' derive from the choices that did not include s_1 as superscript, and similarly for the eight points lying on or close to the line labelled 'not O^2 '. The point labelled 'mean' was calculated from the means of the flux control coefficients; the point labelled 'median' was calculated from the medians of the flux control coefficients adjusted to satisfy the summation relationship by dividing by the sum of the three medians.

large (at least 1000), such rare events do not significantly distort the variations among the 27 combinations.

Figure 4 gives an indication of the scatter of results obtained at the lower error level. Qualitatively, even the worst result out of 100 simulations gives a correct impression of the distribution of flux control, as all of the points without exception indicate that E_2 has the greatest share of flux control, that E_1 has a much smaller share, and that E_3 has almost none. The particular results shown were obtained using $[C_1^J, C_2^J, C_3^J]$ to construct D^J , but we have examined other possibilities, and the qualitative appearance of the figure changes very little on passing from the best to the worst D^J , i.e. from $[C_1^J, C_2^J, C_3^J]$ to $[C_1^J, C_2^J, C_3^J]$.

The reason why different choices of D^J lead to somewhat different control matrices is that they use different sets of co-response coefficients. Although there are nine distinct co-response coefficients for a system of three enzymes (not counting values that are 1 by definition or reciprocals of ones already counted), each choice of D^J uses only six of them. One might hope therefore that the best approach would be to make equivalent use of all nine values. One way to do this would be to calculate all 27 (or n^n in the general case of n enzymes) matrices of control coefficients and take the mean or median of each coefficient as the best value. If the median is used, the resulting control coefficients need to be adjusted to satisfy the summation relationships (see Fell, 1992), because the median is not a linear function of the individual elements, and taking separate medians of n sets of values does not guarantee to preserve any linear relationships that may exist in the original values.

If such averaging is done, one also needs to consider whether to do the averaging when the elasticities are calculated and to calculate the control matrix from averaged elasticities, or to use each elasticity matrix as it is and average the resulting control coefficients. The former approach may have two advantages, of which the second is probably trivial in practice: first, it requires a much smaller number of matrix inversions ($1 + n^n$ instead of $2n^n$); second, the number of elasticities to be averaged is smaller than the number of control coefficients ($n^2 - n$ instead of n^2).

Some results with averaged coefficients are included in Table

3, from which it would appear that means give generally better results than medians (despite the sensitivity of the mean to rare events), and that averaging elasticities is better than averaging control coefficients. The improvement due to averaging is much less than the factor of about 5 that one might naively expect, because the 27 values used in each average are by no means independent, and points derived from different D^v matrices show obvious relationships with one another (Figure 5).

As n^n increases extremely steeply with n (1, 4, 27, 256, 3125, 46656, 823543... for $n = 1, 2, 3, 4, 5, 6, 7...$), it is obvious that any sort of averaging of all possible matrices becomes prohibitive for systems of appreciable size. Nonetheless, it is a realistic option for smaller systems of the kind currently studied in most experimental applications of control analysis. Even with $n = 6$ the cost in computer time would almost certainly be trivial compared with the cost of the experiment itself.

EFFECTS OF SYSTEMATIC ERROR

All of the preceding discussion of effects of experimental error has implicitly assumed that such error is statistical in character. However, one can readily imagine that measurements of the free concentrations of metabolites in a whole system might be reasonably reproducible but subject to large systematic errors, for example one might have measurements reproducible within 10% but systematically in error by a factor of two or more. It is pertinent to ask, therefore, how much effect large systematic errors might have on the type of analysis that we propose. The answer is simple and satisfying: such errors have no effect whatever on the analysis. Although the co-response profiles may be displaced in an unknown direction by an unknown amount from their true positions, their shapes, and hence their slopes at the point of interest, are not affected by systematic multiplicative errors of any magnitude.

SUPERIMPOSABILITY OF CO-RESPONSE PROFILES

It is evident in Figure 2 that two pairs of co-response profiles (the co-responses of s_1 and J to perturbations in E_2 and E_3 , and of s_2 and J to perturbations in E_1 and E_2) not only give the same slopes at the operating point but are actually superimposable. As this behaviour ought to be easy to observe even in systems where it is not possible to do the complete set of perturbations needed for calculating the control matrix, we shall examine what characteristics of the pathway give rise to it and thus how it can be used to diagnose them.

Solving eqn. (4) to produce explicit expressions for the control coefficients in terms of the elasticities yields the following expressions for ${}^{E_2}O_{s_1}^J$ and ${}^{E_3}O_{s_1}^J$:

$${}^{E_2}O_{s_1}^J = \frac{C_2^J}{C_2^{s_1}} = \frac{e_{s_1}^{v_1} e_{s_2}^{v_3} - e_{s_1}^{v_3} e_{s_2}^{v_1}}{e_{s_2}^{v_3} - e_{s_2}^{v_1}} \quad (15)$$

$${}^{E_3}O_{s_1}^J = \frac{C_3^J}{C_3^{s_1}} = \frac{e_{s_1}^{v_1} e_{s_2}^{v_3} - e_{s_1}^{v_3} e_{s_2}^{v_1}}{e_{s_2}^{v_3} - e_{s_2}^{v_1}} \quad (16)$$

Inspection of these shows that the condition for them to be identical (with a value of $e_{s_1}^{v_1}$) is that $e_{s_2}^{v_1} = 0$, i.e. that S_2 should have no feedback effect on the activity of E_1 . By a similar argument, the condition for ${}^{E_1}O_{s_2}^J = {}^{E_2}O_{s_2}^J$ is that $e_{s_1}^{v_3} = 0$, i.e. that there is no feedforward effect of S_1 on E_3 . One should expect, therefore, that addition of feedback inhibition of E_1 by S_2 in the model of Figure 1 would separate the J - s_1 co-response profiles of E_2 and E_3 , but would leave the J - s_2 co-response profiles of E_1 and E_2 superimposable; this is indeed what happens (Figure 6).

Figure 6 Effect of a feedback loop

When feedback inhibition of E_1 by S_2 is added to the model of Figure 1, replacing the first rate equation by $v_1 = e_1(10x_0 - 3s_1)/(2 + x_0 + 2s_1 + 5s_2)$, the superimposed curves in the top-left panel of Figure 2 become separated, but there is no effect on the superimposed curves in the bottom-left panel (which indicate the absence of a feedforward effect of S_1 on E_3).

This interpretation can be generalized into a rule, by analogy with the analysis of eqn. (42) of Hofmeyr and Cornish-Bowden (1991). If a metabolite S_i divides a system into two blocks, so that the enzymes in one block communicate with those in the other only via S_i , then all co-response coefficients $O_{s_i}^J$ for the enzymes of block 1 will be equal. However, if some other metabolite S_j in block 1 has a feedback or feedforward effect on an enzyme in block 2, then all block 1 enzymes on one side of S_j will have the same value of $O_{s_i}^J$, and all the block 1 enzymes on the other side of S_j will have the same value of $O_{s_i}^J$, but the two sets of values will be different. This means that a type of crossover effect will not only reveal the existence of a feedback or feedforward effect, but will also localize the metabolite responsible.

MORE COMPLEX PATHWAYS

For simplicity we have only considered a three-step linear pathway in this study. One may expect that problems of error propagation may increase as the number of enzymes increases, so care is needed before generalizing the encouraging results of our analysis to more complex cases. This is especially true for branched pathways, which will certainly require study in the future, in view of the many branches that exist in metabolism. However, it will probably be better to await additional experimental information on simple pathways before taking the theoretical analysis beyond what current experimental knowledge can support.

DISCUSSION

Freeing the study of the control properties of metabolic systems from its traditional dependence on mechanistic and kinetic studies of the component enzymes has long been a central objective in control analysis. Mechanistic and kinetic information is necessary

Figure 7 Mitochondrial respiration

The Figure shows a co-response profile calculated from data of Wanders (1985; Figure 8 therein) for rat-liver mitochondria perturbed by the addition of various amounts of hexokinase. The original data showed the rate J of oxygen uptake (measured in O/ng-atom of min per mg of protein) varying concomitantly with the extramitochondrial [ATP]/[ADP] ratio. The context in which the work was done is described in Wanders et al. (1984).

for a full description of a system, of course, but, if one is concerned with the performance of a whole system, the essential is not the presence or absence of particular mechanisms but the capacity to respond to changes in the environment. Nevertheless, until now, all applications of control analysis have required study of each enzyme in isolation, to know, as a minimum, how big the perturbation in its activity is. The 'top-down' approach of Brand and co-workers (Hafner et al., 1990; Brown et al., 1990) is to some degree an exception, but it only avoids studying individual enzymes by grouping enzymes into blocks and then treating each block as a catalytic unit; thus if 'enzyme' in the previous sentence were taken to mean 'catalytic unit' it would not be an exception. Recently, however, we suggested a method that would allow all the control information to be deduced from measurement of the effects of perturbations on fluxes and intermediate concentrations (Hofmeyr et al., 1993).

Detailed examination of this method now reveals two surprising aspects. First, it proves to be remarkably insensitive to making the best choice of control coefficients for constructing the diagonal matrix D^* : although the choices are not all equally good, the differences between them are small. For many purposes, therefore, it will be sufficient to choose the particular control coefficients that one wishes to know.

Table 4 Angular errors

The Table shows the relationship between errors of $\pm 2.87^\circ$ or $\pm 5.77^\circ$ in an angle with the errors in the corresponding tangents. When the true value is negative the ranges are exactly the same, apart from signs, as for the corresponding positive value (exemplified in the Table by the entries for 1 and -1). Negative upper limits marked by * indicate values that are beyond infinity rather than below zero. Note that even the smaller angular error of 2.87° is sufficient to generate infinite errors in some of the co-response coefficients in Table 2.

True value	Range for $\pm 2.87^\circ$	Range for $\pm 5.77^\circ$
-1	-1.1 to -0.90	-1.2 to -0.82
0.1	0.05 to 0.15	-0.001 to 0.20
0.2	0.15 to 0.25	0.097 to 0.31
0.5	0.44 to 0.56	0.38 to 0.63
1	0.90 to 1.1	0.82 to 1.2
2	1.8 to 2.3	1.6 to 2.6
3	2.6 to 3.6	2.2 to 4.5
4	3.3 to 5.1	2.8 to 6.9
5	4.0 to 6.7	3.3 to 10
10	6.6 to 20	4.9 to -960^*
20	10 to -7500^*	6.6 to -20^*

The second point is that the precision with which the co-response coefficients need to be known is much less than one might guess (though it may still, of course, be greater than what is achievable in practice). Even the lower level of data error investigated is very large by the standards of most biochemical measurements. Although an angular error of 2.87° corresponds to $\pm 10\%$ if the true value is unity, it corresponds to much larger errors for values that differ appreciably from unity (Table 4). Most of the co-response coefficients in Table 2 have absolute values in the range 0.4 to 2.5, but three of them are very far outside this range and were thus subject to infinite errors in some of the calculations. The method, however, proved remarkably robust to the presence of such errors, and one may hope, therefore, that even if it proves very difficult to obtain experimental values of co-response coefficients with errors less than a factor of two they may still be adequate for the analysis.

A complication arises if the system under study contains moiety-conserved cycles, because then the rows of elasticity matrix that arise from connectivity relationships do not necessarily contain single elasticities; instead they may contain functions of elasticities and ratios of concentrations of moiety-conserved metabolites (Fell and Sauro, 1985; Hofmeyr et al., 1986). As a result the matrix analogous to that in eqn. (9) that results from dividing the lower rows of the inverted co-response coefficient matrix by the top row does not give all the elasticities. Inversion of this matrix does, however, give all the control coefficients, as in eqn. (10).

The need to be able to measure all variables and perturb all enzymes will probably limit the use of our approach to fairly simple systems (though in this respect it is not fundamentally different from other approaches). There are two moderating points, however. First, even if only two variables can be measured and only two enzymes can be perturbed, determination of whether the two resulting co-response profiles are superimposable will reveal some information about the interactions in the system. Systems containing too many enzymes to be easily manageable can be analysed by grouping the enzymes into blocks and treating each block as if it were an enzyme, as in the 'top-down' approach (Hafner et al., 1990; Brown et al., 1990). In our method, provided that each block contains at least one enzyme that can be perturbed, with measurable effects on the part of the

system outside the block, then block elasticities and control coefficients can be obtained without difficulty.

A sceptical reader may question whether even the modest levels of precision that appear necessary can really be achieved in practice and whether all of the variables that need to be measured can in fact be measured. Although co-response coefficients as such do not appear to have been estimated in the past, there are seven examples in Figure 3 of Groen et al. (1983) of plots that would, with a change of coordinate system, become co-response profiles; these show that it is quite feasible to measure the simultaneous responses of eight variables (flux, and the concentrations of glucose 6-phosphate, fructose 1,6-bisphosphate, glycerone phosphate, 3-phosphoglycerate, phosphoenolpyruvate, oxaloacetate and pyruvate) to a perturbation. Actual co-response profiles from this work, albeit without calculation of co-response coefficients, may be found in Figure 4 of Groen and Westerhoff (1990). As a specific example, Figure 7 shows data from the same laboratory [Figure 8 of Wanders (1985)] for the co-response of mitochondrial respiration and the extramitochondrial [ATP]/[ADP] ratio replotted as a co-response profile. All of these examples show that even the methods available a decade ago were quite adequate for providing data of the quality needed for the kind of analysis that we propose.

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APPENDIX

Statistical treatment of co-response profiles

Naive estimation of the slope of a plot of a variable $\log y_2$ against a second variable $\log y_1$ using the standard least-squares calculation would give

$$\hat{O}_{y_1}^{y_2} = \frac{\Sigma [\log(y_1/\bar{y}_1) \log(y_2/\bar{y}_2)]}{\Sigma [\log(y_1/\bar{y}_1)]^2} \quad (\text{A1})$$

in which $\bar{y}_1$ and $\bar{y}_2$ are the geometric means of y_1 and y_2 respectively (i.e. the antilogarithms of the arithmetic means of $\log y_1$ and $\log y_2$). Conversely, the slope of the plot with the axes reversed would be

$$\hat{O}_{y_2}^{y_1} = \frac{\Sigma [\log(y_1/\bar{y}_1) \log(y_2/\bar{y}_2)]}{\Sigma [\log(y_2/\bar{y}_2)]^2} \quad (\text{A2})$$

Note that these slopes are not reciprocals of one another, i.e. $\hat{O}_{y_1}^{y_2} \neq 1/\hat{O}_{y_2}^{y_1}$. The asymmetry arises because the formulae imply that one variable is subject to error whereas the other is not, which is certainly not the case with co-response profiles. The simplest way to treat the variables even-handedly and thereby achieve the desired symmetry is to define the best value of the slope as the geometric mean of $\hat{O}_{y_1}^{y_2}$ and $1/\hat{O}_{y_2}^{y_1}$, i.e.

$$(\tilde{O}_{y_1}^{y_2})^2 = \hat{O}_{y_1}^{y_2} / \hat{O}_{y_2}^{y_1} = \frac{\Sigma [\log(y_2/\bar{y}_2)]^2}{\Sigma [\log(y_1/\bar{y}_1)]^2} \quad (\text{A3})$$

This equation does not have a unique solution (and it will have no solution if $\hat{O}_{y_1}^{y_2}$ and $1/\hat{O}_{y_2}^{y_1}$ have different signs), and the sign must be determined by inspection. Provided that there is a long enough straight region around the steady state of interest to justify fitting a straight line at all, eqn. (A3) should give reasonable results in most cases.

An alternative, more easily adapted to the case where the co-response profile needs to be treated as a curve, is to minimize the sum of squared deviations measured perpendicular to the fitted straight line. If the line is assumed to pass through the centroid $(\log \bar{y}_1, \log \bar{y}_2)$, the best-fit slope is most easily calculated in terms of the angle $\theta_{y_1}^{y_2}$ (corresponding to a slope $\tan \theta_{y_1}^{y_2}$) that the line makes with the y_1 axis. The perpendicular deviation of a point $(\log y_1, \log y_2)$ from the line is

$$\log(y_2/\bar{y}_2) \cos \theta_{y_1}^{y_2} - \log(y_1/\bar{y}_1) \sin \theta_{y_1}^{y_2}.$$

It is then straightforward to show that the angle $\tilde{\theta}_{y_1}^{y_2}$ that minimizes the sum of squares of such deviations is a solution of the following equation:

$$\tan 2\tilde{\theta}_{y_1}^{y_2} = \frac{2 \Sigma \log(y_1/\bar{y}_1) \log(y_2/\bar{y}_2)}{\Sigma [\log(y_1/\bar{y}_1)]^2 - \Sigma [\log(y_2/\bar{y}_2)]^2} \quad (\text{A4})$$

and the co-response coefficient may be calculated as

$$\tilde{O}_{y_1}^{y_2} = \tan \tilde{\theta}_{y_1}^{y_2} \quad (\text{A5})$$

The worst-fit angle is also a solution to eqn. (A4), with two other solutions that are equivalent because $\tan \theta = \tan(\theta + 180^\circ)$. It

ought to be obvious which is the right solution, unless the scatter is so great that y_1 and y_2 are virtually uncorrelated, in which case they will be useless for estimating the co-response coefficient.

If the co-response profile is curved over the whole range (which is likely to prove the usual case) the co-response coefficient should not strictly be estimated as the slope of a straight line at

all. Various kinds of complication arise if one tries to fit a curve by minimizing the sum of squares of perpendicular deviations (for example, the shortest distance from a point to the line may not be unique) but if the curvature is slight one can in principle use iterative methods similar to those used in non-linear regression.