Matrix Method for Determining Steps Most Rate-Limiting to Metabolic Fluxes in Biotechnological Processes

Hans V. Westerhoff
Laboratory of Molecular Biology, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Douglas B. Kell
Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed SY23, 3DA, United Kingdom

Accepted for publication July 7, 1986

The metabolic control theory developed by Kacser, Burns, Heinrich, and Rapoport is briefly outlined, extended, and transformed so as optimally to address some biotechnological questions. The extensions include (i) a new theorem that relates the control of metabolite concentrations by enzyme activities to flux ratios at branches in metabolic pathways; (ii) a new theorem that does the same for the control of the distribution of the flux over two branches; (iii) a method that expresses these controls into properties (the so-called elasticity coefficients) of the enzymes in the pathway; and (iv) a theorem that relates the effects of changes in metabolite concentrations on reaction rates to the effects of changes in enzyme properties on the same rates. Matrix equations relating the flux control and concentration control coefficients to the elasticity coefficients of enzymes in simple linear and branched pathways incorporating feedback are given, together with their general solutions and a numerical example. These equations allow one to develop rigorous criteria by which to decide the optimal strategy for the improvement of a microbial process. We show how this could be used in deciding which property of which enzyme should be changed in order to obtain the maximal concentration of a metabolite or the maximal metabolic flux.

INTRODUCTION

The engineering of microbial cells to improve the production of desirable primary or secondary metabolites is nowadays commonplace, and given the relatively extensive knowledge of the biochemistry of the organisms, it might seem that decisions as to the expression of which gene or genes should be amplified could always be made quite rationally. In practice, the complexity of the relationship between how enzymes behave individually (i.e., enzyme kinetics) and how a *system of enzymes* behaves is sufficient severely to hinder such rational approaches.[1-5]

Although mathematical modeling[1,2,6] has provided some help, most such models have remained too phenomenological to be able to employ the detailed kinetic knowledge of microbial enzymes that may exist for particular cases. Thus, in seeking to improve or intensify a particular fermentation process, it would be most useful to have or to develop simple theorems that might be used to relate the properties of individual enzymes to the steady-state fluxes through metabolic pathways for which they are the catalysts.

To give an adequate answer to many of the questions that might be asked when devising a strategy for optimizing a microbial fermentation, however, may require a less than complete mathematical model. Such questions tend to take the form: To what extent will the rate at which the microorganism produces a certain product increase if the concentration of a certain enzyme is increased somewhat? Recently,[7] we noted that it is exactly this type of question that is being answered with greatly increased effectiveness in the field of intermediary metabolism. The reason for this increased effectiveness lies in the application of the principles of the metabolic control theory of Kacser and Burns[3] and Heinrich and Rapoport.[4]

In this article, we demonstrate algorithms that may be used to deduce from the kinetic properties of enzymes within metabolic pathways which of those enzymes should be increased by genetic manipulation so as most effectively to increase a metabolic flux or the steady-state concentration of a particular metabolite.

Biotechnology and Bioengineering, Vol. XXX, Pp. 101-107 (1987)

CCC 0006-3592/87/010101-07$04.00

SURVEY OF PRINCIPLES OF METABOLIC CONTROL THEORY

Because the metabolic control theory has been reviewed recently,[2,7,8,9] we shall only briefly summarize its major tenets. It considers a metabolic system consisting of enzymes (e) and metabolites (X). Except for pathway substrates (S) and pathway products (P), the concentrations of the metabolites are freely variable (see refs. 2 and 9). In the steady state, however, they and the reaction rates and pathway fluxes take a value determined by the so-called parameters (i.e., the time-invariant properties of the system). Among these parameters we find the concentrations and kinetic constants of the enzymes and the concentrations of pathway substrates, pathway products, and (unmetabolized) external effectors (e.g., added inhibitors). The extent to which a steady state flux J is controlled by any parameter p is parameterized by the flux control coefficient C_p^J:

$$C_p^J \equiv \frac{p}{J}\left(\frac{dJ}{dp}\right)_{ss} = \left(\frac{\mathrm{d}\ln|J|}{\mathrm{d}\ln p}\right)_{ss} \tag{1}$$

where ss refers to the fact that one considers transitions between *steady states* (differing in only one parameter value, p). Similarly, a concentration control coefficient for metabolite X is defined as

$$C_p^X \equiv \frac{p}{[X]}\cdot\left(\frac{d[X]}{dp}\right)_{ss} = \left(\frac{d\ln[X]}{d\ln p}\right)_{ss} \tag{2}$$

There are two summation theorems:

$$\sum_{i=1}^{n} C_{e_i}^J = 1 \tag{3}$$

$$\sum_{i=1}^{n} C_{e_i}^X = 0 \tag{4}$$

where the summation is over all enzymes in the system. In these theorems the parameters p are the e_i's, i.e., the concentrations or activities of the n enzymes of the pathway.

If one of the variable metabolite concentrations, say $[X]$, is altered, there is an instantaneous effect on the rates of any reaction in which X is involved. Such an effect on a reaction rate, v_i, at constant values of all other variables is parameterized by the elasticity coefficients of enzyme e_i with respect to metabolite X:

$$\epsilon_X^{v_i} \equiv \frac{[X]}{v_i}\cdot\frac{\partial v_i}{\partial[X]} = \frac{\partial\ln|v_i|}{\partial\ln[X]} \tag{5}$$

Also a change in any parameter p may have an instantaneous effect on reaction rate v_i, the elasticity coefficient of the enzyme, e_i, with respect to parameter p being defined by

$$\epsilon_p^{v_i} = \frac{p}{v_i}\cdot\frac{\partial v_i}{\partial p} = \frac{\partial\ln|v_i|}{\partial\ln p} \tag{6}$$

The control coefficients are related to the elasticity coefficients through connectivity theorems. The flux control connectivity theorem reads

$$\sum_{i=1}^{n} C_{e_i}^J\cdot\epsilon_{X_k}^{v_i} = 0 \tag{7}$$

The concentration control connectivity theorems[10] state

$$\sum_{i=1}^{n} C_{e_i}^{X_i}\cdot\epsilon_{X_k}^{v_i} = -\delta_k^j \tag{8}$$

with $\delta_k^j = 1$ if $j = k$ and 0 otherwise.

If there is a branch in a pathway, then additional theorems exist at the branch. If flux J branches into two fluxes, J_1 and J_2, then[9]

$$J_2\cdot\sum_{\text{branch1}} C_{e_i}^J - J_1\cdot\sum_{\text{branch2}} C_{e_i}^J = 0 \tag{9}$$

This theorem may be called the flux control branching theorem. We now formulate the anologous concentration control branching theorem for concentration control coefficients:

$$J_2\cdot\sum_{\text{branch1}} C_{e_i}^X - J_1\cdot\sum_{\text{branch2}} C_{e_i}^X = 0 \tag{10}$$

The proof on this theorem is analogous to the proof[9] of equation 9. Let us change the concentrations of all enzymes in branch 1 by the fraction $d\ln a$. This will instantaneously increase the flux J_1 through branch 1 by that same fraction. If simultaneously we decrease the enzyme concentrations in branch 2 by the fraction $J_1(d\ln a)/J_2$, then J_2 will instantaneously decrease by the latter fraction. The change in the total $(J_1 + J_2)$ flux that takes away metabolite X amounts to

$$d(J_1 + J_2) = J_1\cdot d\ln a - J_2\cdot J_1\cdot(d\ln a)/J_2 = 0 \tag{11}$$

Consequently, this concomitant change in the concentrations of the enzymes in the branches does not affect the concentration of metabolite X_4 (Fig. 1). In fact, the concentrations of the other metabolites (and flux J) are not affected either. Since the change in concentration of any metabolite concentration can be expressed in terms of the concentration control coefficients, this conclusion leads to

$$0 = \sum_{\text{branch1}} C_{e_i}^X\cdot d\ln a - \sum_{\text{branch2}} C_{e_i}^X\cdot J_1\cdot(d\ln a)/J_2 \tag{12}$$

which proves equation (10).

If a pathway is branched, then it can be relevant to keep track of the distribution of the total flux over the two branches. To allow this, we here define the flux-branching ratio, j_r, as (for the specific example of Fig. 1)

$$j_r \equiv J_1/J_2 \tag{13}$$

In analogy with equations (1) and (2), we define the flux ratio control coefficient $C_p^{j_r}$ as

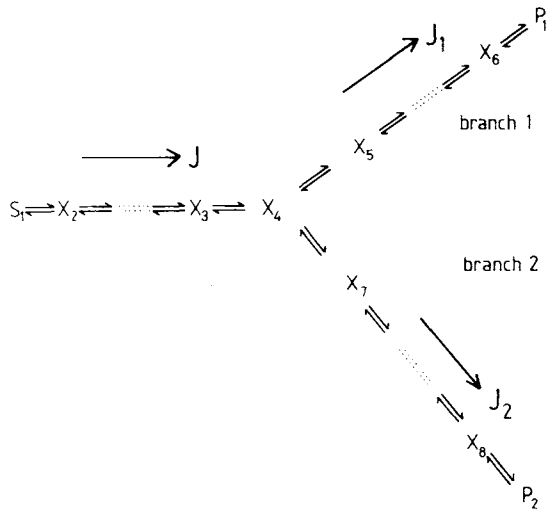

Figure 1. Branched metabolic pathway. Main pathway flux J branches at metabolite X_4 into J_1, flux through branch 1, and J_2, flux through branch 2. S_1, P_1, and P_2: pathway substrates and products present at constant concentration. X_j: metabolites with variable concentrations.

$$C^{j_r}_{p} \equiv d\ln(j_r)/d\ln(p) \tag{14}$$

For the variations in the enzyme activities that were discussed in the preceding paragraph, one finds that

$$\begin{aligned} d\ln(j_r) &= d\ln(J_1) - d\ln(J_2) \\ &= d\ln(a) + (J_1/J_2)\cdot d\ln(a) \end{aligned} \tag{15}$$

By definition, this must also be equal to

$$\begin{aligned} d\ln(j_r) = &\sum_{\text{branch1}} C^{j_r}_{e_i}\cdot d\ln a \\ &+ \sum_{\text{branch2}} C^{j_r}_{e_i}\cdot -\frac{J_1}{J_2}\cdot d\ln a \end{aligned} \tag{16}$$

Combination of equations (16) and (15) yields the flux ratio control branching theorem:

$$\frac{J_2}{J}\cdot\sum_{\text{branch1}} C^{j_r}_{e_i} - \frac{J_1}{J}\cdot\sum_{\text{branch2}} C^{j_r}_{e_i} = 1 \tag{17}$$

The control exerted by an external effector I is related to the control exerted by the enzyme affected by the effector through

$$C^{J}_{I} = C^{J}_{e_i}\cdot\epsilon^{v_i}_{I}$$

and

$$C^{X}_{I} = C^{X}_{e_i}\cdot\epsilon^{v_i}_{I} \tag{19}$$

if e_i is the only enzyme affected by I. If effector I affects more than one enzyme, the products on the right-hand side of equations (18) and (19) are summed over the enzymes i with which the effector interacts.

SOLVING CONTROL STRUCTURE OF PATHWAY

For a linear pathway of n reactions (see Fig. 2 for an example where $n = 4$), the flux control coefficients

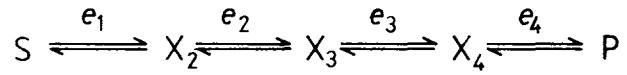

Figure 2. Unbranched metabolic pathway lacking feedback (as well as feed-forward) inhibition (and stimulation). S and P: pathway substrate and pathway product present at constant concentrations. e_j: enzymes. X_j: metabolites present at variable concentrations.

are related through $n - 1$ connectivity theorems [eq. (7), one for the concentration of each metabolite internal to the pathway] and one summation theorem [eq. (3)]. Solution of the n independent linear equations will yield an expression for each flux control coefficient in terms of all elasticity coefficients. Especially in cases of long pathways with feedback or feed-forward stimulation or inhibition, the solution of such equations becomes somewhat tedious. Fell and Sauro[9] have devised the following simple algorithm, which one may readily use to calculate flux control coefficients even if one does not fully appreciate the mathematics of the connectivity and summation theorems. Here, we extend the algorithm so as to include calculation of concentration control coefficients. The first step is to write down an $n \times n$ matrix, M. The first row consists of 1's. The second row of this matrix contains the elasticity coefficients of all the enzymes in the pathway with respect to the first metabolite whose concentration is variable, ordered with respect to the number of the enzyme in the sequence. The third row consists of the elasticity coefficients of all the enzymes with respect to the second variable metabolite. The nth row contains the elasticity coefficients of all the enzymes with respect to the final $(n - 1)$th metabolite concentration (remembering that $[S]$ and $[P]$ are parameters, they are not to be considered here). The second step is to use a computer to invert the matrix M, giving M^{-1}. The first column of M^{-1} now contains the flux control coefficients of the respective enzymes on the pathway flux.

To this procedure, we now add a method to calculate the concentration control coefficients: for the coefficients, $C^{X_3}_{e_i}$, quantifying the control exerted by enzyme i on metabolite X_3, one has

$$\begin{aligned} (C^{X_3}_1 C^{X_3}_2 C^{X_3}_3 C^{X_3}_4 C^{X_3}_5)^T &= M^{-1}\cdot(0 \quad 0 \quad -1 \quad 0 \quad 0)^T \\ &= -(\text{third column of } M^{-1}) \end{aligned} \tag{20}$$

Thus, in general, the ith column of M^{-1} gives minus the concentration control coefficients of the different enzymes for metabolite i (if we start numbering with S_1). The proof of the method (for a good grasp, see the next section) resides in the property of equation (21) (I is the unity matrix, with 1 in all positions on the main diagonal and zero in all other positions):

$$M\cdot C = I \tag{21}$$

that it contains all the summation and connectivity theorems (eqs. (3), (4), (7), and (8)] and that in a linear pathway these completely determine the control structure of the pathway, provided that C is constructed as follows (n enzymes, $n - 1$ metabolites):

$$C = \begin{pmatrix} C_1^J & -C_1^{X_2} & -C_1^{X_3} & \cdots & -C_1^{X_n} \\ C_2^J & -C_2^{X_2} & -C_2^{X_3} & \cdots & -C_2^{X_n} \\ \vdots & \vdots & \vdots & & \vdots \\ C_n^J & -C_n^{X_2} & -C_n^{X_3} & \cdots & -C_n^{X_n} \end{pmatrix} \quad (22)$$

As a consequence (we assume M to be nonsingular[10]),

$$C = M^{-1} \quad (23)$$

(It may be noted that Fell and Sauro[9] only used the first column of this matrix as a column vector. They were only dealing with the flux control coefficients.) Thus, equations (21)–(23) relate all flux and concentration control coefficients to the elasticity coefficients of the enzymes constituting the pathway of interest.

SAMPLE PATHWAY AS ILLUSTRATION

As a more explicit illustration, let us consider the linear pathway of Figure 3, which incorporates feedback inhibition of X_4 on the second enzyme in the pathway. In the algorithm solving the control structure, we constitute the matrix M:

$$M = \begin{pmatrix} 1 & 1 & 1 & 1 \\ \epsilon_{X_2}^1 & \epsilon_{X_2}^2 & \epsilon_{X_2}^3 & \epsilon_{X_2}^4 \\ \epsilon_{X_3}^1 & \epsilon_{X_3}^2 & \epsilon_{X_3}^3 & \epsilon_{X_3}^4 \\ \epsilon_{X_4}^1 & \epsilon_{X_4}^2 & \epsilon_{X_4}^3 & \epsilon_{X_4}^4 \end{pmatrix} \quad (24)$$

As in equation (22), C is defined by

$$C = \begin{pmatrix} C_1^J & -C_1^{X_2} & -C_1^{X_3} & -C_1^{X_4} \\ C_2^J & -C_2^{X_2} & -C_2^{X_3} & -C_2^{X_4} \\ C_3^J & -C_3^{X_2} & -C_3^{X_3} & -C_3^{X_4} \\ C_4^J & -C_4^{X_2} & -C_4^{X_3} & -C_4^{X_4} \end{pmatrix} \quad (25)$$

If we define matrix F by

$$F = M \cdot C \quad (26)$$

then we see that

$$F_{11} = C_1^J + C_2^J + C_3^J + C_4^J \quad (27)$$

According to the flux control summation theorem [eq. (3)], this must equal 1. F_{21} (second row, first column) equals

$$F_{21} = C_1^J \cdot \epsilon_{X_2}^1 + C_2^J \cdot \epsilon_{X_2}^2 + C_3^J \cdot \epsilon_{X_2}^3 + C_4^J \cdot \epsilon_{X_2}^4 \quad (28)$$

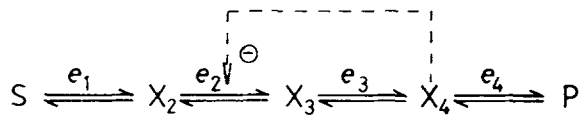

Figure 3. Unbranched metabolic pathway with feedback inhibition. ⊖, Inhibition of enzyme 2 by X_4.

According to the flux control connectivity theorem [eq. (7)], this must equal zero.

$$F_{33} = -C_1^{X_3} \cdot \epsilon_{X_3}^1 - C_2^{X_3} \cdot \epsilon_{X_3}^2 - C_3^{X_3} \cdot \epsilon_{X_3}^3 - C_4^{X_3} \cdot \epsilon_{X_3}^4 \quad (29)$$

The concentration control connectivity theorem [eq. (8), $k = j$] requires that $F_{33} = 1$. Similarly,

$$F_{23} = -C_1^{X_3} \cdot \epsilon_{X_2}^1 - C_2^{X_3} \cdot \epsilon_{X_2}^2 - C_3^{X_3} \cdot \epsilon_{X_2}^3 - C_4^{X_3} \cdot \epsilon_{X_2}^4 \quad (30)$$

must be zero. Then

$$F_{13} = -C_1^{X_3} - C_2^{X_3} - C_3^{X_3} - C_4^{X_3} \quad (31)$$

should equal zero due to the concentration control summation theorem [eq. (4)]. Working one's way through all the elements of F, one finds that its main diagonal elements are equal to 1, whereas all its other elements equal zero. Thus, $F = MC$ equals the identity matrix, which proves equation (21). Thus, equation (23) solves for the control coefficients in terms of the elasticity coefficients.

Most often, many elements of M equal zero. This simplifies the calculations somewhat. For the pathway of Figure 3, where the only "distant" effect of metabolites is the feedback inhibition of e_2 by X_4,

$$\epsilon_{X_3}^1 = \epsilon_{X_4}^1 = \epsilon_{X_2}^3 = \epsilon_{X_2}^4 = \epsilon_{X_3}^4 = 0 \quad (32)$$

Matrix M is then simplified to

$$\mathrm{M} = \begin{pmatrix} 1 & 1 & 1 & 1 \\ \epsilon_{X_2}^1 & \epsilon_{X_2}^2 & 0 & 0 \\ 0 & \epsilon_{X_3}^2 & \epsilon_{X_3}^3 & 0 \\ 0 & \epsilon_{X_4}^2 & \epsilon_{X_4}^3 & \epsilon_{X_4}^4 \end{pmatrix} \quad (33)$$

BRANCHED PATHWAYS

For a branched pathway, the number of metabolites becomes less than the number of enzymes, so that the above procedure will not produce a square matrix M. Fell and Sauro[9] derived a theorem [eq. (9)] that, together with our equations (10 and 17), provides the equations required for the missing rows of M. We illustrate this for the branched pathway given by Figure 4:

$$\mathrm{M} = \begin{pmatrix} 1 & 1 & 1 & 1 & 1 \\ \epsilon_{X_2}^1 & \epsilon_{X_2}^2 & 0 & 0 & 0 \\ 0 & \epsilon_{X_3}^2 & \epsilon_{X_3}^3 & 0 & \epsilon_{X_3}^5 \\ 0 & \epsilon_{X_4}^2 & \epsilon_{X_4}^3 & \epsilon_{X_4}^4 & 0 \\ 0 & 0 & j_5 & j_5 & j_5 - 1 \end{pmatrix} \quad (34)$$

where j_5 is the fraction of J that flows through the branch containing enzyme 5. The first row, responsible for the summation theorems, again consists of 1's. Rows 2–4 of M again consist of an enumeration of the elasticity coefficients of all five enzymes with respect to all three metabolites. Upon multiplication with C [eq. (22)], these columns generate the connectivity theorems. The fifth row embodies equations (9) and (10). In this last row, each element that corresponds to an

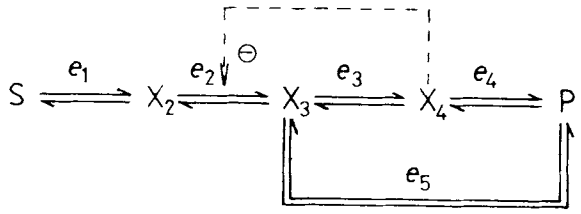

Figure 4. Branched metabolic pathway with feedback inhibition. Pathway flux J, which flows through enzymes e_1 and e_2 branches at X_3 into flux $J{\cdot}j_5$, which flows through enzyme e_5 and flux $J{\cdot}(1 - j_5)$, which flows through enzymes e_3 and e_4.

enzyme not in either branch (but in the unbranched part of the pathway) becomes zero. The other elements equal 1 *minus* the fraction of pathway flux J that flows through the enzyme.

The solution [eq. (37)] is further analogous to the unbranched case discussed above. Here C is defined as in equation (22), except that an extra column contains the control coefficients that refer to the control exerted by the enzymes on the branch ratio j_r:

$$C = \begin{pmatrix} C_1^J & -C_1^{X_2} & -C_1^{X_3} & -C_1^{X_4} & C_1^{jr} \\ C_2^J & -C_2^{X_2} & -C_2^{X_3} & -C_2^{X_4} & C_2^{jr} \\ C_3^J & -C_3^{X_2} & -C_3^{X_3} & -C_3^{X_4} & C_3^{jr} \\ C_4^J & -C_4^{X_2} & -C_4^{X_3} & -C_4^{X_4} & C_4^{jr} \\ C_5^J & -C_5^{X_2} & -C_5^{X_3} & -C_5^{X_4} & C_5^{jr} \end{pmatrix} \quad (35)$$

the equation that contains the summation theorems and connectivity theorems as well as the branching theorems [eqs. (9) and (10)] is

$$M{\cdot}C = \begin{pmatrix} 1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 1 \end{pmatrix} \quad (36)$$

so that equation (23) again gives the magnitudes of all control coefficients, now including those that refer to the flux ratio at the branch (j_r). The reader may wish to check that the bottom elements of MC correspond to the flux and concentration control branching theorems [eqs. (9) and (10)]. Also, the last column of M·C contains the analogues of equations (4) and (7) for j_r as well as equation (17).

NUMERICAL EXAMPLE OF SOLVING CONTROL STRUCTURE

As a numerical example, let us consider the case (Fig. 2) without feedback inhibition ($\epsilon^2_{X_4} = 0$), with the elasticities specified taking the values [corresponding to the M of eq. (24)] in the following matrix:

$$M = \begin{pmatrix} 1 & 1 & 1 & 1 \\ -0.9 & 0.5 & 0 & 0 \\ 0 & -0.2 & 0.7 & 0 \\ 0 & 0 & -0.1 & 0.9 \end{pmatrix} \quad (37)$$

Inverting matrix M, one finds

$$\begin{pmatrix} C_1^J & -C_1^{X_2} & -C_1^{X_3} & -C_1^{X_4} \\ C_2^J & -C_2^{X_2} & -C_2^{X_3} & -C_2^{X_4} \\ C_3^J & -C_3^{X_2} & -C_3^{X_3} & -C_3^{X_4} \\ C_4^J & -C_4^{X_2} & -C_4^{X_3} & -C_4^{X_4} \end{pmatrix} = M^{-1}$$

$$= \begin{pmatrix} 0.30 & -0.78 & -0.48 & -0.33 \\ 0.53 & 0.59 & -0.85 & -0.59 \\ 0.15 & 0.17 & 1.19 & -0.17 \\ 0.02 & 0.02 & 0.13 & 1.09 \end{pmatrix} \quad (38)$$

For this particular example, all the flux control coefficients lie between 0 and 1. Most flux control (i.e., 53%) lies in enzyme 2; this enzyme is a sort of "bottleneck." Yet, enzymes 1 and 3 also exert significant flux control. It is also seen here that concentration control coefficients tend to be positive when the enzyme precedes the controlled metabolite and negative when it succeeds it. Enzyme 3 has rather strong negative control on the concentration of metabolite X_3 (a 1% increase in the former decreases the latter by 1.19%).

As a second numerical example, we consider a case where there is significant feedback inhibition by X_4 on e_2: $M(2, 4) = \epsilon^2_X = -1$. Keeping the other elasticity coefficients at the same magnitudes as in equation (37), we find, for the control coefficients [by inverting M as in eq. (38)],

$$\begin{pmatrix} C_1^J & -C_1^{X_2} & -C_1^{X_3} & -C_1^{X_4} \\ C_2^J & -C_2^{X_2} & -C_2^{X_3} & -C_2^{X_4} \\ C_3^J & -C_3^{X_2} & -C_3^{X_3} & -C_3^{X_4} \\ C_4^J & -C_4^{X_2} & -C_4^{X_3} & -C_4^{X_4} \end{pmatrix}$$

$$= \begin{pmatrix} 0.19 & -0.90 & -0.30 & -0.21 \\ \underline{0.34} & \underline{0.37} & -0.53 & \underline{-0.37} \\ 0.10 & 0.11 & 1.28 & -0.11 \\ \underline{0.38} & \underline{0.43} & \underline{-0.45} & 0.69 \end{pmatrix} \quad (39)$$

where we have underlined the values that have greatly changed as a consequence of the feedback inhibition. Most importantly, although enzyme 2 is now strongly feedback inhibited, its flux control coefficient has significantly *decreased*. It turns out that flux control has shifted toward enzyme 4. Another striking feature is that the control exerted by enzyme 4 on X_2 is now strongly negative: An increased activity of enzyme 4 will lead to a significant *decrease* in $[X_2]$. An activator (external effector) of enzyme 4 would cause a "false" crossover because it would cause a decrease in X_2 and an increase in X_3, i.e., a crossover at enzyme 2. One might be misled and conclude that the activator activates enzyme 2 rather than enzyme 4.

The matrix method used here is very effective if approximate numerical values for the elasticity coefficients are available. In cases where such *a priori* knowledge is absent, it may still be useful to solve the connectivity and summation theorem equations analytically. For the flux control coefficients in Figure 3, we obtain [through the application of eqs. (3) and (7) and tedious but straightforward algebra]

$$C_1^J = \rho_2 \cdot \rho_3 \cdot \rho_4 / \Sigma \quad (40)$$

$$C_2^J = \rho_3 \cdot \rho_4 / \Sigma \quad (41)$$

$$C_3^J = \rho_4 / \Sigma \quad (42)$$

$$C_4^J = (1 + \rho_4 \cdot \epsilon_4^2 / \epsilon_4^3) / \Sigma \quad (43)$$

with

$$\Sigma = \rho_2 \cdot \rho_3 \cdot \rho_4 + \rho_3 \cdot \rho_4 + \rho_4 + 1 + \rho_4 \cdot \epsilon_4^2 / \epsilon_4^3 \quad (44)$$

and

$$\rho_i \equiv \epsilon_{X_i}^i / - \epsilon_{X_i}^{i-1} \quad (45)$$

where ρ_i is minus the ratio of the two elasticity coefficients with respect to X_i, i.e., minus the one of the enzyme for which X_i is the substrate divided by the one of the enzyme for which X_i is the product. In "featureless" pathways ($\epsilon_4^2 = 0$), the ρ's exceed 1 and hence the flux control decreases from the first enzyme down to the last [cf. eqs. (40)–(45)]. The present equations clearly show that strong feedback inhibition by X_4 can greatly increase the flux control of enzyme 4 and decrease the flux control exerted by the preceding enzymes.

ANSWERING BIOTECHNOLOGICAL QUESTIONS

When engineering a cell that is used to excrete a valuable substance, the relevant question is: How should we modify which enzymes to increase the rate at which that substance is excreted? If, on the other hand, the cells are harvested and lysed so as to isolate the desirable product, then the question is: How should we modify which enzymes in order to increase the "steady-state" concentration (either in a continuous culture or in the "idiophase" of a batch culture) of the metabolite of interest? The first column of matrix C bears on the former question(s), whereas the other columns bear on the latter. However, some additional aspects of the problem need to be considered.

First, we must consider which parameters of our system are likely to be adjustable through genetic manipulation. An obvious one is the concentration of the enzymes. If we can vary the concentration of enzyme i by the (small) fraction $\delta \ln[e_i]$ (e.g., by increasing the strength of the promoter of the structural gene for that enzyme), then the change in flux will be approximately equal to $C_{e_i}^J \cdot \delta \ln[e_i]$ (δ refers to a small change). If we can vary catalytic activity (by varying k_{cat}), by $\delta \ln[k_{cat}]$, then, very similarly, the effect on the flux will be $C_{e_i}^J \cdot \delta \ln[k_{cat}]$.

One may also alter the way in which an enzyme interacts with one of its substrates, products, or effectors. In almost all rate equations for enzyme-catalyzed reactions, the concentrations of such substances occur in combination with (actually in a ratio to) a constant, either a K_m , a K_i, or a K_d. It is this constant that may again be subject to alteration through genetic modification of the enzyme. If indeed the metabolite concentration X_j only occurs in the equation as a ratio to the constant K_j, then

$$\epsilon_{K_j}^{v_i} = -\epsilon_{X_j}^{v_i} \quad (46)$$

The proof of this relationship is found by writing the reaction rate as $v(y)$ with $y = X_j/K_j$, then taking the two partial derivatives with respect to X_j and K_j, respectively, and using the chain rule

$$\frac{\partial v_i}{\partial X_j} = \frac{\partial v_i}{\partial y} \cdot \frac{1}{K_j} \quad (47)$$

$$\frac{\partial v_i}{\partial K_j} = \frac{\partial v_i}{\partial y} \cdot \frac{-1}{(K_j)^2} \cdot X_j \quad (48)$$

Thus, it is readily shown that

$$\epsilon_{K_j}^i = \frac{K_j}{v_i} \cdot \frac{\partial v_i}{\partial K_j} = \frac{-X_j}{K_j v_j} \cdot \frac{\partial v_i}{\partial y} = \frac{-X_j}{K_j v_i} \cdot K_j \cdot \frac{\partial v_i}{\partial X_j} = \frac{-X_j}{v_i} \cdot \frac{\partial v \mathrm{yi}}{\partial X_j} = -\epsilon_{X_j}^i \quad (49)$$

For an alteration of K_j by a (small) fraction $\delta \ln K_j$, the resulting effect on the steady-state flux will be $d \ln J = -C_{e_i}^J \cdot \epsilon_{X_j}^{v_i} \cdot \delta \ln K_j$.

To evaluate which alteration is going to be the most effective in changing flux J, one may construct the matrix B. Here B is identical to M except that every elasticity coefficient is multiplied with the change in corresponding K and the l's are replaced with the fractional change in activity of the corresponding enzyme. If C_{diag}^J is defined as

$$C_{\mathrm{diag}}^J \equiv \begin{pmatrix} C_{e_1}^J & 0 & 0 & \cdots & 0 \\ 0 & C_{e_2}^J & 0 & \cdots & 0 \\ 0 & 0 & C_{e_3}^J & \cdots & 0 \\ \vdots & \vdots & \vdots & & \vdots \\ 0 & 0 & 0 & \cdots & C_{e_n}^J \end{pmatrix} \quad (50)$$

then the matrix A given by

$$A \equiv B \cdot C_{\mathrm{diag}}^J \quad (51)$$

consists of elements that are each characteristic for an effect of a change in a parameter of the system on flux J. If we are looking for the best strategy to increase the flux, then the largest element of A gives the parameter change we are looking for, in the sense that it will lead to the greatest change in flux for the given percentage change in K_m, K_d, K_i, k_{cat}, or $[e_i]$.

Let us consider the pathway of Figure 3 by way of an example. Our matrix B becomes

$$B = \begin{pmatrix} \delta \ln e_1 & \delta \ln e_2 & \delta \ln e_3 & \delta \ln e_4 \\ -\epsilon_{X_2}^1 \cdot \delta \ln K_2^1 & -\epsilon_{X_2}^2 \cdot \delta \ln K_2^2 & 0 & 0 \\ 0 & -\epsilon_{X_3}^2 \cdot \delta \ln K_3^2 & -\epsilon_{X_3}^3 \cdot \delta \ln K_3^3 & 0 \\ 0 & -\epsilon_{X_4}^2 \cdot \delta \ln K_4^2 & -\epsilon_{X_4}^3 \cdot \delta \ln K_4^3 & -\epsilon_{X_4}^4 \cdot \delta \ln K_4^4 \end{pmatrix} \quad (52)$$

Here K_j^i refers to the Michaelis, inhibition, etc., constant of enzyme i with respect to metabolite X_j.

The C^J_{diag} is obtained from equation (50):

$$C^J_{\text{diag}} = \begin{pmatrix} C_1^J & 0 & 0 & 0 \\ 0 & C_2^J & 0 & 0 \\ 0 & 0 & C_3^J & 0 \\ 0 & 0 & 0 & C_4^J \end{pmatrix} \tag{53}$$

Thus, for A we obtain

$$A = \begin{pmatrix} C_1^J \cdot \delta \ln e_1 & C_2^J \cdot \delta \ln e_2 & C_3^J \cdot \delta \ln e_3 & C_4^J \cdot \delta \ln e_4 \\ -C_1^J \cdot \epsilon^1_{X_2} \cdot \delta \ln K_2^1 & -C_2^J \cdot \epsilon^2_{X_2} \cdot \delta \ln K_2^2 & 0 & 0 \\ 0 & -C_2^J \cdot \epsilon^2_{X_3} \cdot \delta \ln K_3^2 & -C_3^J \cdot \epsilon^3_{X_3} \cdot \delta \ln K_3^3 & 0 \\ 0 & -C_2^J \cdot \epsilon^2_{X_4} \cdot \delta \ln K_4^2 & -C_3^J \cdot \epsilon^3_{X_4} \cdot \delta \ln K_4^3 & -C_4^J \cdot \epsilon^4_{X_4} \cdot \delta \ln K_4^4 \end{pmatrix} \tag{54}$$

If all parameters changes would amount to 2%, our numerical example would become

$$100 \cdot A = \begin{pmatrix} 0.19 & 0.34 & 0.10 & 0.38 \\ -0.17 & 0.17 & 0 & 0 \\ 0 & -0.07 & 0.07 & 0 \\ 0 & -0.34 & -0.01 & 0.34 \end{pmatrix} \tag{55}$$

The largest elements of A are those corresponding to changing $[e_4]$ (0.38), $[e_2]$, K_4^2, and K_4^4 (all ± 0.34). Thus, for this example the best strategy would be to increase the concentration of e_4. Almost equally good would be the strategies of increasing $[e_2]$, increasing the K_m of this enzyme for its feedback inhibitor, X_4, or *decreasing* the K_m of enzyme 4 for its substrate.

It also follows that strategies altering the product inhibition of enzyme 3 (-0.01), product inhibition of enzyme 2 (-0.07), or the K_m of e_3 for its substrate (0.07) are rather undesirable. If the changes in the parameters that we can produce are different from each other (i.e., not all equal to 2%), then the elements of matrix A should first be multiplied by the achievable change before determining which element is greatest.

If one is interested in the maximization of a metabolite *concentration* (say, X_3) rather than a flux, then the C^J_{diag} in the above analysis should be replaced with C^X_{diag}, which is obtained from the third row of M^{-1}. The further analysis is identical.

It should be noted, strictly speaking, that the above analysis is only valid when one is considering small changes in the parameters. For large changes (say > 10%), the predictions of the method become less reliable, but on the average, they will still be better than predictions obtained from nonsystematic methods.

Similarly, the above analysis strictly applies to stationary steady state, though microbial growth, of course, may sometimes have additional properties.[11,12]

DISCUSSION

In this article, we have shown how the metabolic control theory derived for biochemical systems by Kacser and Burns[3] and Heinrich and Rapoport[4] may be tailored to address questions that may be asked while devising strategies for the genetic manipulation of productive microbial strains. The result is a relatively straightforward matrix method that, with the present ubiquity of microcomputers, leads to a simple expression of the effects of genetic changes in enzyme properties on steady-state fluxes and metabolic concentrations in terms of the kinetic properties of the cellular complement of enzymes. Of course, this does not alleviate the need for knowledge of those kinetic properties but at least provides for a *rational* choice between potential schemes for the manipulation of microbial enzymes based on whatever information is available. That limitations in the application of metabolic control theory in the case of productive microbes pose no significant problem of principle has recently been discussed elsewhere.[7]

D. B. K. thanks the Biotechnology Directorate of the Science and Engineering Research Council, U.K., for financial support. We thank Ms. Mary Lou Miller for the expert typing of the manuscript.

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