Quantitative analysis of metabolic regulation

A graph-theoretic approach using spanning trees

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A graph-theoretic technique using spanning trees is described for the evaluation of Flux Control Coefficients of metabolic pathways. The technique is illustrated by investigating a linear pathway (a) in the absence of feedback and feedforward regulation, (b) with its first enzyme inhibited by the end product and (c) with multiple feedback loops. It is shown that the Flux Control Coefficients of a linear pathway with one or more feedback loops can be derived in a systematic manner by superimposing the effect of the feedback loop(s) on the expressions pertaining to the Flux Control Coefficients of the unregulated pathway.

INTRODUCTION

Quantitative analysis of metabolic regulation is an active area of current research [1]. There are three principal avenues along which metabolic control analysis is being carried out. They are called Biochemical Systems Theory [2–4], Flux-Oriented Theory [5–7] and Metabolic Control Theory [8,9]. Among them, Metabolic Control Theory has enjoyed the most popularity in recent years. Several similarities and differences in these theories have been pointed out recently by Savageau *et al.* [10–13] and Cascante *et al.* [14,15]. In Metabolic Control Theory the regulatory effect of an enzyme on a metabolic flux (or a metabolite concentration) is described by a Flux Control Coefficient (or a Concentration Control Coefficient). Two theorems, known as Summation and Connectivity Theorems, are devised to formulate the problem for the Control Coefficients of the enzymes in terms of their Elasticity Coefficients. These theorems lead to a system of linear algebraic equations; their solution yields the expressions for the Control Coefficients. Of the various methods proposed for the solution of these equations, the matrix-inversion technique [16,17] is commonly used by researchers. A diagrammatic method described by Hofmeyr [18] is also available for control-pattern analysis of metabolic pathways. Within the premises of Metabolic Control Theory we recently presented a graph-theoretic approach, based on signal flow graphs, for the calculation of the Control Coefficients [19]. In our related work [20,21], we introduced the idea of using electrical analogues for evaluating these Control Coefficients. The purpose of the present paper is to develop an alternate graph-theoretic approach for the analysis of metabolic regulation. The present graph-theoretic approach consists of finding the spanning trees in a weighted directed graph representing the control structure of the metabolic pathway. This is distinct from the method of signal flow graphs used previously [19], which requires enumeration of the loops or cycles in the corresponding signal flow graph. The directed graphs to be used here also possess a structure very different from the signal flow graphs. The concept of spanning trees was used by King & Altman [22] about 35 years ago and has been subsequently extended for the analysis of steady-state enzyme kinetics and other biochemical systems [23–27]. Graph-theoretic techniques have also been used for investigating the stability properties of multienzyme systems [28]. Here we shall apply the method of spanning trees, with appropriate modifications, for the cal-

ulation of Flux Control Coefficients of metabolic pathways. Analogous to the method of signal flow graphs, the main advantage of this approach is that the Control Coefficients of the enzymes can be evaluated in terms of their Elasticity Coefficients, without the necessity of writing down the Summation and Connectivity Theorems. We shall illustrate this graph-theoretic technique by investigating a linear pathway (a) in the absence of feedback and feedforward regulation, (b) with its first enzyme inhibited by the end product and (c) with multiple feedback loops. In the Appendix a mathematical justification is given of the method of spanning trees as it applies to the calculation of the Control Coefficients. For the sake of simplicity, we shall restrict our considerations to the Flux Control Coefficients.

AN UNREGULATED LINEAR PATHWAY

Consider the following linear pathway, with no feedback or feedforward regulation:

It contains five enzymes, E_1 – E_5 , an initial precursor, X_1 , and four variable metabolites, X_2 , X_3 , X_4 and X_5 . In order to determine the Flux Control Coefficients of the enzymes, we first construct a directed graph, simply called a digraph, characterizing the control structure of the pathway. Construction of the digraph is facilitated by redrawing the pathway with the Elasticity Coefficients of the various enzymes displayed explicitly as shown below.

We used a similar representation previously [19–21]. Here an Elasticity Coefficient ϵ_j^i is indicated by a broken line with an arrow pointing from the metabolite X_j to the enzyme E_i . Using this representation the digraph of Fig. 1(a) is constructed in the following manner. The nodes 1–5 are introduced to designate the enzymes E_1 – E_5 and their unknown Flux Control Coefficients C_i ($i = 1, 2, 3, 4, 5$). To connect these nodes, we first focus our attention on the segment of the pathway (A1) containing only

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Fig. 1. (a) A digraph for the unregulated pathway (A), (b) the spanning tree to node 2 in the digraph of (a) and (c) the spanning tree to node 5 in the digraph of (a)

Fig. 2. (a) A digraph for pathway (B), (b) the digraph of (a) after deletion of the edges (4,5) and (5,4) and (c) the spanning tree to node 5 in (b)

the enzymes E_1 and E_2 and their adjoining metabolite, X_2 . Consider the Elasticity Coefficient ϵ_2^1 , which points in the reverse (i.e., upstream) direction of the pathway. Corresponding to this Elasticity Coefficient, a directed edge is drawn from node 1 to node 2 in Fig. 1(a), carrying the weight $-\epsilon_2^1 = \bar{\epsilon}_2^1$ (where $\epsilon_2^1 = -\bar{\epsilon}_2^1$ with $\bar{\epsilon}_2^1 > 0$). For the Elasticity Coefficient ϵ_2^2 , which points in the forward (i.e., downstream) direction of the pathway, we have drawn an edge from node 2 to node 1 in Fig. 1(a) with the weight ϵ_2^2 . In general, the various nodes in Fig. 1(a) are joined by considering each segment of the pathway containing a contiguous pair of enzymes and their shared metabolite, and adopting the following sign convention. In the representation (A1), if an Elasticity Coefficient ϵ_i^j points in the reverse direction of the pathway, then an edge (i, j) is drawn from node i to node j in the digraph, carrying the weight $-\epsilon_i^j$. On the other hand, for an Elasticity Coefficient ϵ_i^j pointing in the forward direction of the pathway, an edge is directed from node i to node $(i-1)$ with the weight ϵ_i^j . Furthermore, since ϵ_i^j is an Elasticity Coefficient of product inhibition and is negative, we have written $\epsilon_i^j = -\bar{\epsilon}_i^j$, $\bar{\epsilon}_i^j$ being the absolute value of the Elasticity Coefficient.

From Fig. 1(a) the Flux Control Coefficient, C_i , of an enzyme E_i can be derived with the aid of the formula:

$$C_i = N_i / \sum_{k=1}^5 N_k, \quad i = 1, 2, 3, 4, 5 \quad (1)$$

The quantity N_i is defined as follows: form the tree product (i.e. the product of the edge-weights) in each **spanning tree** to node i in the digraph; N_i is given by the sum of these tree products. A **tree** is a connected subgraph which contains no loops or cycles. A spanning tree is a tree passing through every node in the digraph [29]. As an example, let us evaluate C_2 . Observe that in Fig. 1(a) there is only one spanning tree to node 2; this is displayed in Fig. 1(b). Multiplying the edge-weights in this tree, we find $N_2 = \bar{\epsilon}_2^1 \epsilon_3^3 \epsilon_4^4 \epsilon_5^5$. Similarly, in Fig. 1(a) there is a spanning

tree to node 5, as shown in Fig. 1(c). From this Figure we obtain $N_5 = \bar{\epsilon}_2^1 \epsilon_3^3 \epsilon_4^4 \epsilon_5^5$. The remaining quantities, namely, N_1 , N_3 and N_4 , can be found in an analogous fashion. The Flux Control Coefficients of the various enzymes can then be deduced from eqn. (1). For example, the Flux Control Coefficient of enzyme E_5 is given by the expression:

$$C_5 = \frac{\bar{\epsilon}_2^1 \epsilon_3^3 \epsilon_4^4 \epsilon_5^5}{\bar{\epsilon}_2^1 \epsilon_3^3 \epsilon_4^4 \epsilon_5^5 + \bar{\epsilon}_2^1 \epsilon_3^3 \epsilon_4^4 \epsilon_5^5 + \bar{\epsilon}_2^1 \epsilon_3^3 \epsilon_4^4 \epsilon_5^5 + \bar{\epsilon}_2^1 \epsilon_3^3 \epsilon_4^4 \epsilon_5^5 + \bar{\epsilon}_2^1 \epsilon_3^3 \epsilon_4^4 \epsilon_5^5} \quad (2)$$

Spanning trees can also be used for investigating the cause-effect relationships of the individual enzymes. For instance, observe in Fig. 1(b) that, if $\bar{\epsilon}_2^1 = 0$, i.e., if the enzyme E_1 is devoid of product inhibition, the edge (1,2) will have zero weight and may therefore be deleted. Under this condition there will be no spanning tree to node 2; as a result C_2 will be zero. It is also apparent from Fig. 1(b) that, if any of the Elasticity Coefficients ϵ_3^3 , ϵ_4^4 or ϵ_5^5 is zero, i.e., if any of the enzymes E_3 , E_4 or E_5 is saturated, then C_2 must be zero. In general, by enumerating the spanning trees to each node in Fig. 1(a), it can be shown that, in pathway (A), (i) all enzymes which lie downstream from an enzyme without product inhibition have a zero Flux Control Coefficient; the control of flux is distributed among the enzyme with no product inhibition and those located upstream from it; (ii) all enzymes situated upstream from a saturated enzyme exert no control on the flux; the flux is regulated by the saturated enzyme and those lying downstream from the saturated enzyme. These conclusions, which are otherwise intuitively clear, were deduced previously [19] with the aid of signal-flow graphs. Although such extreme conditions are not found *in vivo*, their analysis may be useful in establishing the trend in the overall systemic behaviour.

A LINEAR PATHWAY WITH FEEDBACK INHIBITION

We now examine a linear pathway in which the activity of the first enzyme is inhibited by the end product:

The Flux Control Coefficients of this pathway can be derived from eqn. (1) by writing $N_i = N_i^0 + N_i^a$, where the zero superscript refers to the unregulated pathway and the superscript 'a' indicates the contribution due to feedback inhibition. This feedback contribution can be assessed by modifying the digraph of Fig. 1(a) as follows. We redraw the pathway (B) by deleting the feedback loop and all the enzymes (and metabolites) which are located inside the loop, thereby treating the enzymes E_1 and E_5 as contiguous enzymes with X_5 as their adjoining metabolite.

As usual, the relevant Elasticity Coefficients are shown by broken lines in the above representation. Here e_s^1 is the Elasticity Coefficient of enzyme E_1 due to feedback inhibition. In view of the fact that e_s^1 points in the upstream direction of the pathway, we draw an edge from node 1 to node 5 in Fig. 1(a) with a weight $-e_s^1$, in accordance with the convention adopted in the previous section. But since e_s^1 is negative, we write $e_s^1 = -\bar{e}_s^1$ (with $\bar{e}_s^1 > 0$), so that the directed edge (1,5) carries the weight $\bar{e}_s^1$, as shown in Fig. 2(a). To evaluate N_i^a for any enzyme E_i , we first delete the edges that are incident on node 5 to and from the immediately preceding node, i.e., node 4; see Fig. 2(b). Next we locate all the spanning trees to node i in Fig. 2(b) and form the product of the edge-weights in each spanning tree; N_i^a is equal to the sum of these products. Since there are no spanning trees to any of the nodes 1, 2, 3 or 4 in Fig. 2(b), it follows that $N_i^a = 0$ for $i = 1, 2, 3$ or 4. There is, however, a spanning tree to node 5 in this Figure as portrayed in Fig. 2(c); from its tree product, we find $N_5^a = e_2^2 e_3^3 e_4^4 \bar{e}_s^1$. As a result, C_5 is given by:

$$C_5 = (N_5^0 + N_5^a) / (N_1^0 + N_2^0 + N_3^0 + N_4^0 + N_5^0 + N_5^a)$$

which amounts to:

$$C_5 = \frac{\bar{e}_s^1 e_2^2 e_3^3 e_4^4 + e_2^2 e_3^3 e_4^4 e_s^1}{e_2^2 e_3^3 e_4^4 e_s^5 + \bar{e}_s^1 e_2^2 e_3^3 e_4^4 e_s^5 + \bar{e}_s^1 e_2^2 e_3^3 e_4^4 e_s^5 + \bar{e}_s^1 e_2^2 e_3^3 e_4^4 e_s^5 + \bar{e}_s^1 e_2^2 e_3^3 e_4^4 e_s^5 + \bar{e}_s^1 e_2^2 e_3^3 e_4^4 e_s^5} \quad (3)$$

For the first four enzymes we have $C_i = N_i^0 / D$, $i = 1, 2, 3, 4$, with D being equal to the denominator on the right-hand side of eqn. (3). The cause-effect relationships of the various enzymes in this pathway can be analysed in the same fashion as in the unregulated pathway; for the sake of brevity, this will not be discussed here.

LINEAR PATHWAYS WITH MULTIPLE FEEDBACK LOOPS

When multiple feedback loops are present, their effect can be superimposed on the expressions pertaining to the Flux Control Coefficients of the unregulated pathway. To see how this is done, consider first a linear pathway with two overlapping feedback loops. In particular, consider the pathway shown below in which the enzyme E_1 undergoes feedback inhibition by the metabolites X_3 and X_5 :

Fig. 3(a) depicts the digraph for this pathway. This Figure is drawn from Fig. 1(a) by adding the directed edges (1,5) and (1,3)

corresponding to the feedback loops (a) and (b) respectively. For deriving the Flux Control Coefficients of the enzymes, we first examine the effect of the two feedback loops separately. The effect of the outer feedback loop (a) is the same as that of the feedback loop in pathway (B). To account for the inner feedback loop (b), we ignore the edge (1,5) and delete the edges (2,3) and (3,2), which connect node 3 to and from the immediately preceding node, i.e., node 2 in Fig. 3(a); this gives rise to Fig. 3(b). It is easy to see that, in Fig. 3(b), there is a spanning tree to each of the nodes 3, 4 and 5; these are portrayed in Figs. 3(c), 3(d) and 3(e) respectively. From the product of the edge-weights in the individual trees, the contribution of the inner feedback loop is given by $N_3^b = e_2^2 e_3^1 e_4^4 e_5^5$, $N_4^b = e_2^2 e_3^1 e_4^3 e_5^5$ and $N_5^b = e_2^2 e_3^1 e_4^3 e_5^4$. Next we examine the 'cumulative' effect of the two feedback loops (a) and (b) by deleting the edges (4,5), (5,4), (2,3) and (3,2) from Fig. 3(a), as shown in Fig. 3(f). Clearly since there are no spanning trees to any of the nodes in this Figure, the two feedback loops do not 'interact' to yield a term containing both Elasticity Coefficients e_s^1 and e_s^5 . We therefore have $N_1 = N_1^0$, $N_2 = N_2^0$, $N_3 = N_3^0 + N_3^b$, $N_4 = N_4^0 + N_4^b$, and $N_5 = N_5^0 + N_5^a + N_5^b$. Accordingly, the Flux Control Coefficients of this pathway can be expressed as $C_i = N_i / \bar{D}$, for $i = 1, 2, 3, 4, 5$, with $\bar{D} = N_1 + N_2 + N_3 + N_4 + N_5$.

A linear pathway in which the same metabolite inhibits two different enzymes is considered next. More specifically, we examine the following pathway where the enzymes E_1 and E_3 are inhibited by the end product:

A digraph for this pathway, which is shown in Fig. 4(a), is obtained from Fig. 1(a) by inserting the edges (1,5) and (3,5). From this Figure the Flux Control Coefficients of the enzymes are evaluated as follows. The contribution of the outer feedback loop (a) is the same as that found with pathway (B). To assess the effect of the inner feedback loop (c), we remove the edge (1,5) from Fig. 4(a) and subsequently delete the edges (4,5) and (5,4), which are incident on node 5 to and from the preceding node, i.e., node 4. The resulting digraph is depicted in Fig. 4(b). We see that there is only one spanning tree in this Figure; it is directed to node 5 (see Fig. 4(c)). The product of the edge-weights in this tree yields $N_5^c = \bar{e}_s^2 e_3^3 e_4^4 e_5^5$, which should be added to N_5^0 . Finally, we use the following procedure to see if there is a cumulative effect of the two feedback loops. Note that, in Fig. 4(a), the edges (1,5) and (3,5), representing the two feedback loops, are both directed to the same node (i.e., node 5). We delete the edges connecting node 5 to its preceding node, i.e., node 4; we also delete the edges which are incident on node 4 from its preceding node, i.e. node 3. This procedure makes node 4 an isolated node, thereby disconnecting the digraph as shown in Fig. 4(d). Clearly since no spanning trees can exist in Fig. 4(d), it follows that the two feedback loops *jointly* do not contribute to the expressions for the Flux Control Coefficients. In other words, there cannot be any terms involving the product $\bar{e}_s^2 e_s^3$ in any of the expressions for the Flux Control Coefficients. The Flux Control Coefficients are then given by $C_i = N_i^0 / \bar{D}$, $i = 1, 2, 3, 4$, and $C_5 = (N_5^0 + N_5^a + N_5^c) / \bar{D}$, where $\bar{D} = N_1^0 + N_2^0 + N_3^0 + N_4^0 + N_5^0 + N_5^a + N_5^c$.

There are two other configurations with an overlapping pattern of feedback loops. These arise when two enzymes are inhibited by two different metabolites and (i) the feedback loops lie one inside the other as in pathway (E); (ii) the two feedback loops are located in an overhanging fashion, as in pathway (F).

Fig. 3. (a) A digraph for pathway (C), (b) the digraph of (a) after deletion of the edges (1,5), (2,3) and (3,2), (c) the spanning tree to node 3 in (b), (d) the spanning tree to node 4 in (b), (e) the spanning tree to node 5 in (b) and (f) the digraph of (a) after deletion of the edges (2,3), (3,2), (4,5) and (5,4)

Fig. 4. (a) A digraph for pathway (D), (b) the digraph of (a) after deletion of the edges (1,5), (4,5) and (5,4), (c) the spanning tree to node 5 in (b) and (d) the digraph of (a) after deletion of the edges (4,5), (5,4), (3,4) and (4,3)

whereas the digraph for pathway (F) consists of Fig. 1(a) with two additional edges (2,4) and (3,5). It can be easily seen that, in either pathway, there will be no 'cumulative' contribution of the two feedback loops to the expressions for the Flux Control Coefficients.

Finally we examine a linear pathway with two non-overlapping feedback loops as shown below. The feedback loops in this pathway have no enzyme or metabolite in common.

The treatment of these pathways is similar to the pathways described above. For instance, the digraph for pathway (E) is obtained by inserting the edges (1,5) and (2,4) in Fig. 1(a),

Fig. 5. (a) A digraph for pathway (G), (b) the digraph of (a) after deletion of the edges (2,3), (3,2), (4,5) and (5,4) and (c) the spanning tree to node 5 in (b)

The digraph for this pathway is given in Fig. 5(a), which is constructed from the digraph of the unregulated pathway (Fig. 1a) with the addition of the edges (1,3) and (3,5). To calculate the Flux Control Coefficients we start by considering the feedback loops, one at a time. The effect of the left feedback loop (b) is the same as that described earlier with pathway (C), and the effect of the right feedback loop (c) is identical with that in pathway (D). Next the cumulative effect of the two feedback loops is analysed by deleting from Fig. 5(a) the two pairs of edges (2,3), (3,2) and (4,5), (5,4); see Fig. 5(b). The only spanning tree in this Figure is directed to node 5, and is displayed in Fig. 5(c). From this Figure the cumulative effect of the feedback loops is found to be $N_5^{bc} = e_2^2 e_3^1 e_4^2 e_5^3$, which contains the Elasticity Coefficients of both feedback loops. Collectively, we obtain $N_1 = N_1^0$, $N_2 = N_2^0$, $N_3 = N_3^0 + N_3^b$, $N_4 = N_4^0 + N_4^b$ and $N_5 = N_5^0 + N_5^b + N_5^c + N_5^{bc}$, so that the Flux Control Coefficients are given by $C_i = N_i/D^*$, for $i = 1, 2, 3, 4, 5$, with $D^* = N_1 + N_2 + N_3 + N_4 + N_5$.

The results obtained in this section may be summarized as follows. For a linear pathway with two feedback loops, designated (α) and (β), the Flux Control Coefficient of an enzyme E_i can be deduced from eqn. (1), with $N_i = N_i^0 + N_i^\alpha + N_i^\beta + N_i^{\alpha\beta}$. Here the superscript zero, as usual, stands for the unregulated pathway; the superscripts α and β refer to the contribution of the individual feedback loops (α) and (β), and the superscript $\alpha\beta$ indicates the cumulative effect of the two feedback loops. The quantity N_i^α or N_i^β is non-zero for every enzyme E_i located downstream from the feedback loop (α) or (β) respectively; for all other enzymes in the pathway, $N_i^\alpha = N_i^\beta = 0$. The cumulative term $N_i^{\alpha\beta}$ is non-zero *only* when the two feedback loops do not overlap, and the enzyme E_i lies downstream of the rightmost feedback loop. These results can be easily generalized when the pathway contains more than two feedback loops.

CONCLUDING REMARKS

Using the method of spanning trees we have described a systematic procedure for evaluating the Flux Control Coefficients of a linear pathway with and without feedback loops. It is shown that the Flux Control Coefficients of a linear pathway with feedback inhibition can be derived by superimposing the effect of

the feedback loops on the expressions pertaining to the Flux Control Coefficients of the unregulated pathway. The present methodology can be extended to more complex pathways such as those with a branched or a cyclic structure.

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APPENDIX

We show below how the directed graphs presented in the main paper can be constructed from a matrix formulation of the governing equations for the Flux Control Coefficients and thereby provide a mathematical justification of the method of spanning trees for evaluating these Control Coefficients. For this purpose we begin with the unregulated pathway (A). The Flux Control Coefficients of the enzymes in this pathway are governed by the matrix equation $\mathbf{M} \cdot \mathbf{C} = \mathbf{b}$, i.e.:

$$\begin{bmatrix} 1 & 1 & 1 & 1 & 1 \\ -\bar{e}_2^1 & e_2^2 & 0 & 0 & 0 \\ 0 & -\bar{e}_3^2 & e_3^3 & 0 & 0 \\ 0 & 0 & -\bar{e}_4^3 & e_4^4 & 0 \\ 0 & 0 & 0 & -\bar{e}_5^4 & e_5^5 \end{bmatrix} \begin{bmatrix} C_1 \\ C_2 \\ C_3 \\ C_4 \\ C_5 \end{bmatrix} = \begin{bmatrix} 1 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \quad (\text{A1})$$

From the matrix $\mathbf{M}$, the directed graph shown in Fig. 1(a) can be constructed as follows. We first draw five nodes representing the unknown Flux Control Coefficients of the five enzymes. These nodes are connected by directed edges using the following convention. Only the Elasticity Coefficients appearing in $\mathbf{M}$ are designated in Fig. 1(a); the 1's in the first row of $\mathbf{M}$ are not used in this digraph. If $M_{ij} \neq 0$, then an edge is drawn in Fig. 1(a) from node j to node i carrying the weight $-M_{ij}$; if $M_{ii} \neq 0$, we draw an edge from node i to node $(i-1)$ with the weight M_{ii} .

Using Cramer's rule the Flux Control Coefficient of an enzyme E_i can be found from eqn. (A1) with the aid of the formula $C_i = \Delta_i / \Delta$, $i = 1, 2, 3, 4, 5$, where Δ_i is the determinant of the matrix formed by replacing the i th column of $\mathbf{M}$ by the vector $\mathbf{b}$, and Δ is the determinant of $\mathbf{M}$. Furthermore, since $C_1 + C_2 + C_3 + C_4 + C_5 = 1$, it follows that $\Delta_1 + \Delta_2 + \Delta_3 + \Delta_4 + \Delta_5 = \Delta$. consider, as an example, the evaluation of C_5 . This Flux Control Coefficient is given by $C_5 = \Delta_5 / \Delta$, where:

$$\Delta_5 = \begin{vmatrix} 1 & 1 & 1 & 1 & 1 \\ -\bar{e}_2^1 & e_2^2 & 0 & 0 & 0 \\ 0 & -\bar{e}_3^2 & e_3^3 & 0 & 0 \\ 0 & 0 & -\bar{e}_4^3 & e_4^4 & 0 \\ 0 & 0 & 0 & -\bar{e}_5^4 & 0 \end{vmatrix} = \bar{e}_2^1 \bar{e}_3^2 \bar{e}_4^3 \bar{e}_5^4 \quad (\text{A2})$$

As pointed out in the main paper, there is a spanning tree to node 5 in the digraph of Fig. 1(a); see Fig. 1(c). It is now easily

seen that the product of the edge-weights of this tree is equal to the determinant Δ_5 given in eqn. (A2) above. In general, it can be established that the determinant Δ_i , $i = 1, 2, 3, 4, 5$, is equal to the tree-product of the spanning tree to node i ($i = 1, 2, 3, 4, 5$) in Fig. 1(a). The determinant Δ is the sum of the tree-products of all the spanning trees present in Fig. 1(a). In other words the determinant Δ_i is the same as the quantity N_i defined in eqn. (1) in the main paper.

Next we consider the pathway (B) which possesses feedback inhibition. The Flux Control Coefficients of the enzymes in this pathway are governed by eqn. (A1) if the bottom-left-corner element $M_{51} = 0$ is replaced by $M_{51} = -\bar{e}_5^1$, which is the Elasticity Coefficient of enzyme E_1 due to feedback inhibition. Let us evaluate C_5 in this case. We have $C_5 = \Delta_5^* / \Delta^*$, with:

$$\Delta_5^* = \begin{vmatrix} 1 & 1 & 1 & 1 & 1 \\ -\bar{e}_2^1 & e_2^2 & 0 & 0 & 0 \\ 0 & -\bar{e}_3^2 & e_3^3 & 0 & 0 \\ 0 & 0 & -\bar{e}_4^3 & e_4^4 & 0 \\ -\bar{e}_5^1 & 0 & 0 & -\bar{e}_5^4 & 0 \end{vmatrix} \quad (\text{A3})$$

We have used the superscript * to indicate that these quantities apply to pathway (B), which has feedback inhibition. The determinant Δ_5^* can be expressed as the sum $\Delta_5 + \Delta_5^f$, where Δ_5 is given in eqn. (A2), and:

$$\Delta_5^f = \begin{vmatrix} 1 & 1 & 1 & 1 & 1 \\ -\bar{e}_2^1 & e_2^2 & 0 & 0 & 0 \\ 0 & -\bar{e}_3^2 & e_3^3 & 0 & 0 \\ 0 & 0 & -\bar{e}_4^3 & e_4^4 & 0 \\ -\bar{e}_5^1 & 0 & 0 & 0 & 0 \end{vmatrix} = e_2^2 e_3^3 e_4^4 \bar{e}_5^1 \quad (\text{A4})$$

Note that the matrix associated with the determinant Δ_5^f can be represented by Fig. 2(b), in accordance with the convention adopted earlier in this Appendix. It is also apparent that the determinant Δ_5^f is equal to the tree-product of the spanning tree to node 5 in Fig. 2(b); see Fig. 2(c).

For a linear pathway with multiple feedback loops, the results obtained with spanning trees can be substantiated by constructing a determinant such as Δ_5^* and expressing it as the sum of several determinants, each of which equals the tree-product of a spanning tree in the appropriate digraph.