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Extending Double Modulation: Combinatorial Rules for Identifying the Modulations Necessary for Determining Elasticities in Metabolic Pathways*

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The double modulation method for determining the elasticities of pathway enzymes, originally devised by Kacser & Burns (*Biochem. Soc. Trans.* **7,** 1149–1160, 1979), is extended to pathways of complex topological structure, including branching and feedback loops. An explicit system of linear equations for the unknown elasticities is derived. The constraints imposed on this linear system imply that modulations of more than one enzyme are not necessarily independent. Simple combinatorial rules are described for identifying without using any algebra the set of independent modulations that allow the determination of the elasticities of any enzyme. By repeated application, the minimum numbers of modulations required to determine the elasticities of all enzymes of a given pathway can be determined. The procedure is illustrated with numerous examples.

1. Introduction

In recent years the impression may have emerged that control analysis is becoming little more than a sort of skilful juggling with partial derivatives of fluxes, reaction rates and metabolite concentrations, without much impact on experimental issues. One of the reasons has certainly been the lack of general methods for measuring control coefficients in intact organelles, but the recent appearance of methods of determining elasticities *in vivo* by modulating the activities of all (Hofmeyr *et al*., 1993; Cornish-Bowden & Hofmeyr, 1994) or some (Giersch, 1994, 1995) of the enzymes in the intact system has brought experimental aspects of control analysis more into the focus of interest. These papers have shown that elasticities can be determined without knowing the magnitude of the modulation of a reaction rate brought about by a modulator, provided that it acts on one enzyme only. Experimental interest has thus shifted to the question of numbers and localization within the pathway of reactions that are to be modulated: how many and which reactions have to be modulated to define all elasticities in a given pathway?

Kacser & Burns (1979) showed that modulation of the activities of the first and last enzymes of a chain allows determination of the elasticities of all of the enzymes if there are no feedback loops. Their method, later known as the "double modulation method", was put on a more rigorous footing in work that showed that determination of the elasticities for any enzyme in such a simple chain required two modulations, one upstream and one downstream of the enzyme concerned (Giersch, 1994; Small, 1988).

In this contribution, the double modulation approach is extended to more complex pathways with branch points, regulatory loops, and conserved metabolites. The mathematics allows calculation of the elasticities from an explicit system of linear equations and is thus considerably simpler than an earlier approach (Giersch, 1994), in which an implicit system had to be analysed. The algorithm provides

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lists of all combinations of modulations that allow determination of the elasticities of any enzyme in a given pathway. By analysing several typical pathway configurations, rules are established to derive the combinations of modulations with reference only to the graph of the pathway and without using any mathematical formalism. Repeated application of the combinatorial rules to the individual pathway enzymes allows one to see how many, and which enzymes have to be modulated in order to determine the elasticities of all enzymes.

2. Symbolism and Examples

2.1. LINEAR PATHWAY WITHOUT REGULATORY LOOPS

Figure 1 depicts a simple chain without regulatory loops. Metabolites are denoted by capital letters in alphabetical order, beginning with A (and omitting E), and their concentrations will be represented by the corresponding italic letters; enzymes are denoted by lower-case letters, ending with z. The letter e will be used for an unspecified enzyme. This symbolism has the advantage that it is short, that it avoids the use of double indices, and that the ordering of elements is obvious. However, to avoid confusing metabolites with enzymes it is inappropriate for pathways with more than about 12 metabolites or enzymes, and is also less convenient for more abstract mathematical presentation of the ideas, and for that reason is not used in Section 5.

Earlier work (Giersch, 1994) showed that the elasticities of the enzymes at the ends of the chain (and of all enzymes whose rate is influenced by only one metabolite) can be determined by modulating just one enzyme. For this reason, such modulations are always independent (see Section 5, Mathematical background), and enzymes that depend on one metabolite only will not be considered in detail. Determination of the elasticities of an enzyme whose activity depends on more than one metabolite, such as enzyme x in Fig. 1, requires modulation of two enzymes, one on each side of x: possible pairs of enzymes in this case are w and y or w and z. We denote these combinations here as "(w) (yz)", with the covention that "(set_1) (set_2)" means a list of all possible combinations of one element of set_1 and one element of set_2. Thus, all possible combinations of two enzymes whose modulation will allow determination of the elasticities of y (Fig. 1), are, in this symbolism, given by (wx) (z). Although this symbolism allows one also to recognize the enzyme to which the decomposition applies as the enzyme missing from the expression, y in this case, it is often helpful to indicate this more directly and to give the number of required modulations in square brackets. Thus, for the pathway of Fig. 1, the modulations that allow determination of the elasticities for enzymes x and y can be fully expressed as x[2]: (w) (yz); y[2]: (wx) (z).

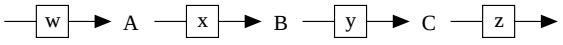

FIG. 1. Simple metabolic chain with metabolites A, B and C, and enzymes w, x, y and z. The enzymes are represented by boxes within reaction arrows. The arrows are not intended to imply that the reactions are irreversible, but to define which direction of reaction corresponds to positive values for the rates. The source and sink metabolites are assumed to be constant reservoirs and are not depicted in the figure.

2.2. CHAIN WITH REGULATORY LOOP

In the pathway of Fig. 2, enzyme v is assumed to be subject to feedback control by metabolite G (in addition to the effects of its direct product D and substrate C). How many modulations are required to determine the elasticity of enzyme w, for example, and which combinations of the enzymes s . . . z can be chosen for the determination?

For any enzyme the number of modulations required is equal to the number of non-zero elasticities, i.e. the number of metabolites that affect the reaction rate. For enzyme w in Fig. 2, it is two (as it is for most of the other enzymes, apart from enzyme v, for which it is three, and enzymes s and z, for which it is one each). Thus to determine the elasticities of w, two enzymes have to be selected. Proper candidates are found as follows:

(1) Omit the enzyme for which the elasticity is to be determined (here w) from the list of pathway reactions (s t u v · x y z) and group the remaining enzymes into two subgroups:

(s t u v) (x y z)

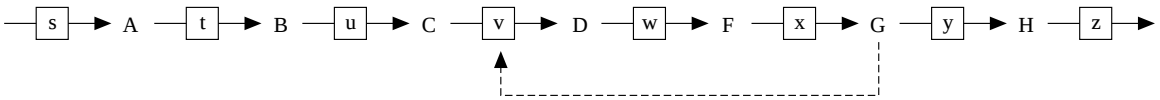

FIG. 2. Metabolic chain with a feedback loop. To derive the rules for decomposing the sequence of enzymes by studying various possible configurations of the feedback loop a more than minimum length of the chain in required. Combinations of possible modulations (symbolized as defined in the text) that allow determinations of the elasticities of each enzyme are as follows: t[2]: (s) (uvwxyz); u[2]: (st) (vwxyz); v[3]: (stu) (wx) (yz); w[2]: (stuv) (x) (yz); x[2]: (stuvw) (yz); y[2]: (stuvwx) (z). Notice that there are two combinations of three enzymes (s, w, z or s, x, z) that allow the determination of all the elasticities.

(2) If the omitted enzyme (w) is located within a regulatory loop, separate the subgroup at the position of the effector of the loop (G in the case of Fig. 2), between the enzymes that produce and consume it, x and y respectively:

(s t u v) (x) (y z).

Note that this second decomposition is required only for enzymes located within the regulatory loop. For enzyme u, for example, which is located upstream of the loop, the two subgroups are (s t) (v w x y z), because no such decomposition of a subgroup is required.

(3) Although there are now three groups, it remains true that two modulations suffice for determining the elasticities of enzyme w; in fact, any two enzymes from different groups of (s t u v) (x) (y z) will allow determination of the elasticities of w. There are $4 \times 3 + 1 \times 2 = 14$ such combinations altogether, i.e. sx, sy, sz; tx, ty, tz; ux, uy, uz; vx, vy, vz; xy, xz. Note that the combinations xy or xz, in which both enzymes are located downstream of w, are independent modulations. On the other hand, combinations of enzymes belonging to the same subgroup, such as st, tu or yz, do not allow determination of the elasticities.

For determining the elasticities of enzyme v, the same procedure produces the decomposition v[3]: (s t u) (w x) (y z), and as modulations of three enzymes are required to determine these elasticities, any of the following $3 \times 2 \times 2 = 12$ triplets of enzymes will suffice: swy, swz, sxy, sxz, twy, twz, txy, txz, uwy, uwz, uxy, uxz. The decompositions for the other enzymes of the pathway are listed in the legend to Fig. 2.

An analogous rule holds for the analysis of pathways with a feedforward loop: to identify the modulations allowing determination of the elasticities of enzyme e, omit e from the enzyme sequence; if e is located within the loop, make a further decomposition at the position of the effector of the loop.

2.3. BRANCHED PATHWAY

In a simple branched pathway as illustrated in Fig. 3, two independent modulations suffice for determining the elasticities of any enzyme, as no enzyme is influenced by more than two metabolites. For example, which combinations of two enzymes allow the elasticities of y to be determined? We may proceed as before, with the steps numbered as in Section 2.2:

(1) Delete enzyme y and collect the subgroups. Here it is essential to note that a branch point as such does not give rise to an additional subgroup, and so enzymes u, v, w and x all belong to the same subgroup: y[2]: (u v w x) (z).

(2) There are no feedback loops.

(3) The four combinations of two enzymes that can be modulated to allow determination of the elasticities of enzyme y are therefore uz, vz, wz, xz. Each of these includes the downstream enzyme z and one enzyme on the other side of y, regardless of whether it is upstream or downstream of the branch-point metabolite B.

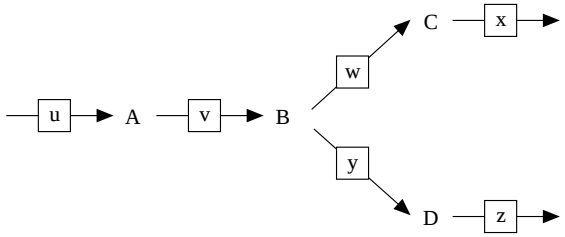

FIG. 3. Typical branch point configuration in a metabolic network. Possible combinations are as follows: v[2]: (u) (wxyz); w[2]: (uvyz) (x); y[2]: (uvwx) (z). Although the elasticities of any single enzyme can be determined from only two modulations, at least three enzymes must be modulated to determine all of them.

3. Control Analytical Background

It has been known for some time that elasticities can be determined by modulating the activities of certain enzymes (Kacser & Burns, 1979; Groen *et al*., 1982). A necessary condition is that the modulations are *independent*, i.e. that the effect on the metabolite levels of a given modulation cannot be substituted for by combinations of other modulations. That independence of modulations of two enzymes is not always guaranteed has been shown before for the simple chain (Giersch, 1994); it is also evident from the "superimposability of co-response profiles" (Cornish-Bowden & Hofmeyr, 1994), and from the possibility of defining "modules" (Rohwer *et al*., 1994) that can be regarded as lists of interdependent modulations.

For a simple chain without feedback loops, we know that independence is always guaranteed if one modulation is upstream and one downstream from the enzyme whose elasticities are to be determined. In extending the modulation approach to more complex pathways, two questions have to be answered: (i) what is the minimum number $M_{\min(e)}$ of modulations required for determining the elasticities of enzyme e, and (ii) which of the possible combinations of $M_{\min(e)}$ enzymes represent independent modulations?

It is not difficult to see that $M_{\min(e)}$ for any enzyme e is equal to the number of metabolites that directly influence the rate of the enzyme, i.e. the number of

nonzero elasticities. Restricting attention for the moment to enzymes with one substrate and one product, this number is two for any enzyme within the pathway that is not subject to regulatory effects, but it is increased by one for each regulatory metabolite that acts on it and is decreased by one if the enzyme in question is at one of the limits of the pathway. For enzymes with more than one substrate or product, it increases by one for each such substrate or product.

It is less obvious how to identify the particular sets of $M_{\min(e)}$ enzymes that provide independent modulations for a given enzyme e. An earlier approach (Giersch, 1994) was partially successful; considering additional equations allows those that contain unmeasurable quantities $\partial v_i/\partial p_i$ (see Section 5) to be omitted, and leaves sets of equations that are relatively simple. These simpler equations allow the problem to be solved and can be used to formulate rules for identifying the sets of independent modulations by inspecting the graph of the metabolic network, without using the usual algebra of metabolic control analysis. In essence, the rules describe how to decompose the sequence of enzymes into subsets in such a way that it is obvious which modulations are independent and which are not. The examples discussed above illustrated that feedback loops cause the decomposition of a subset, whereas branch points do not. The rules have been derived by generalizing from individual cases rather than from a general theory (to derive these "decomposition rules" in closed analytical form from the equations given below will provide a rewarding exercise in linear algebra). We have checked that the rules are valid for the examples considered here, and we are convinced that they will be valid for most (if not all) other situations. In the following, additional rules are presented and exemplified, and subsequently the mathematical background is provided to allow the reader to analyse other, and more complex, networks.

4. More Complex Cases

4.1. TWO INTERCONNECTED FEEDBACK LOOPS

A pathway with two interconnected regulatory loops [Fig. 4(a)] can be analysed according to the decomposition rule given above if another rule to be stated below is added. First we note that any enzyme except z and w require two independent modulations. Determination of the elasticities of w and z require three and one modulations respectively.

Applying the rule that for enzymes within a feedback loop there is an additional decomposition at the position of the effector of the loop, the decomposition for determining the elasticities of u, for example, would be (v w) (x y z), as u is located within the feedback loop C→u but upstream of the loop F→w. However, the correct decomposition can be calculated to be u[2]: (v w) (x y) (z), which means that enzyme u "sees" the loop F→w whose target enzyme w is located within the same feedback loop as enzyme u. Thus, there are $2 \times 3 + 2 \times 1 = 8$ combinations of two enzymes to be modulated that will allow determination of the elasticities of u. Likewise, the decomposition for determining the elasticities of enzyme v is v[2]: (u) (w) (x y) (z), so that there are $1 \times 4 + 1 \times 3 + 2 \times 1 = 9$ combinations of two enzymes to be modulated. For enzyme w, the decomposition is w[3]: (u v) (x y) (z), and $2 \times 2 \times 1 = 4$ combinations of three enzymes can be used to determine the elasticities of w. Note, however, that the decomposition for determining the elasticities

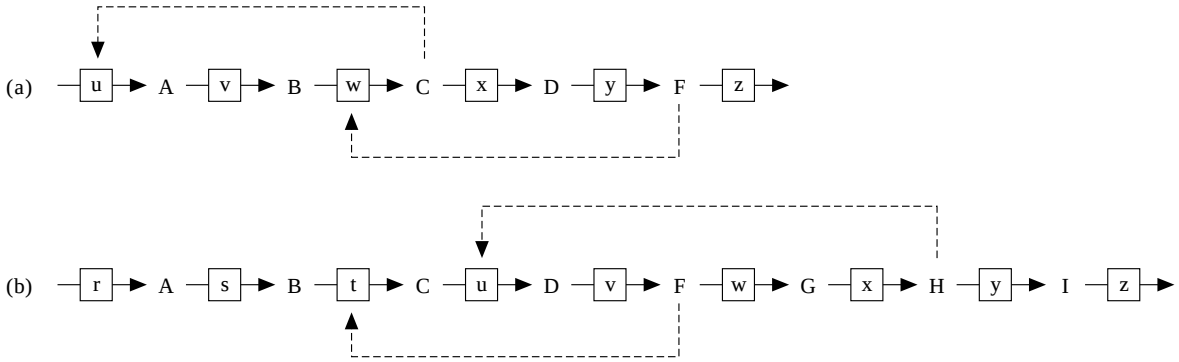

FIG. 4. Two metabolic chains with two interconnected feedback loops, for (a) a simple case, and (b) a more complex case with eight metabolites and nine enzymes. Possible combinations for example (a) are as follows: u[2]: (vw) (xy) (z); v[2]: (u) (w) (xy) (z); w[3]: (uv) (xy) (z); x[2]: (uvw) (y) (z); y[2]: (uvwx) (z). As in Fig. 3, three enzymes have to be modulated to determine all elasticities, though modulation of enzymes w and z allows determination of all elasticities except those of enzyme w. Possible combinations for example (b) are as follows: s[2]: (r) (tuvwxyz); t[3]: (rs) (uv) (wx) (yz), with one of the three modulated enzymes necessarily either r or s; u[3]: (rst) (v) (wx) (yz), with one of the three modulated enzymes necessarily either y or z; v[2]: (rstu) (wx) (yz); w[2]: (rstuv) (x) (yz); x[2]: (rstuvw) (yz); y[2]: (rstuvwx) (z).

of y is calculated to be (u v w x) (z), and not (u v w) (x) (z), which means that enzyme y does not "see" the loop C→u, whose target enzyme u is not located within the same loop as enzyme y. This analysis shows that two interconnected feedback loops (target enzyme but not the effector of the downstream loop located within the upstream loop) cause the decomposition of the sequence of enzymes to be as for one single loop with the exception that enzymes in the upstream loop "see" the downstream loop, whereas those in the downstream loop do not "see" the upstream loop.

Even for the relatively simple case of a pathway with two interconnected regulatory loops a large number of configurations can be imagined: one loop located completely within the other, the two loops starting or ending at the same position, or combinations of a feedback and a feedforward loop. Most if not all of such configurations can be analysed using the above rules and observing an additional rule, which can best be outlined by considering the example in Fig. 4(b), which is a little more complex than that of Fig. 4(a).

The decompositions for determining the elasticities of t and u are t[3]: (rs) (uv) (wx) (yz) and u[3]: (rst) (v) (wx) (yz), respectively. However, not all combinations of three enzymes resulting from these decompositions are independent. Thus, the combination u, w, y does not allow determination of the elasticities of enzyme t although the decomposition t[3]: (rs) (uv) (wx) (yz) suggests that it should. Rather, to determine the elasticities of t, either r or s must always be among the modulated enzymes, and to determine the elasticities of u, either y or z must always be modulated. Such additional rules come into play if three modulations are required and the number of subgroups in which the sequence of enzymes is decomposed exceeds three. For the last example, the additional rule means that to determine the elasticities of t one of the modulated enzymes has to be upstream of the upstream loop, and that to determine the elasticities of u one of the modulated enzymes has to be downstream of the downstream loop. If one of the feedback loops is located completely within the other, determination of the elasticities of the target enzyme of the inner loop requires one of the modulated enzymes to be located within the inner loop. The complete decomposition of this example is given in the legend to Fig. 4.

4.2. CONSERVED METABOLITE CYCLE

Figure 5 depicts a situation in which the sum of two metabolite concentrations $D + F$ is constant. This constraint on the variables reduces the number of independent variables and, therefore, also the rank of matrix $\partial \mathbf{S}/\partial \mathbf{p}$: see Section 5, Mathematical background. However, the system of Fig. 5 can be analysed if one of the variables F is eliminated ($F = \text{const.} - D$), and the following decomposition is calculated: x[2]: (w) (yzv); y[3]: (wx) (z) (v).

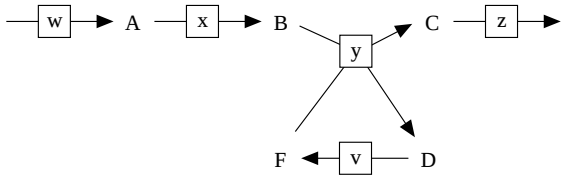

FIG. 5. Metabolic chain in which the sum $D + F$ of the concentrations of co-substrate D and co-product F of enzyme y is conserved.

4.3. PATHWAY WITH BRANCH POINT AND FEEDBACK LOOPS

The pathway depicted in Fig. 6 has one branch point and three regulatory loops. It has been used elsewhere (Cornish-Bowden *et al*., 1995) in numerical simulations to illustrate the effects of regulatory mechanisms on control distribution, and their implications for genetic manipulation of metabolic pathways for biotechnological purposes. The grouping of the enzymes follows the straightforward application of the rules given above, observing that the three feedback loops are not interconnected. Thus, for determining the elasticities of u, the following decomposition applies: u[3]: (s t x y z) (v) (w). Decompositions for determining the elasticities of the other enzymes are listed in the legend to Fig. 6.

Scanning these decompositions it can be seen that modulations of at least six enzymes are required to determine all elasticities of the pathway, and that there is just one such combination, namely s, t, v, w, y, z.

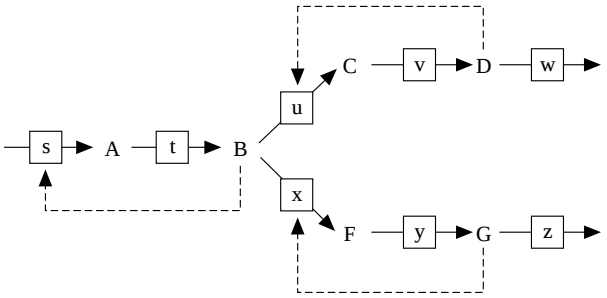

FIG. 6. Metabolic pathway with one branch point and three feedback loops. Possible combinations are as follows: s[2]: (t) (uvwxyz); t[2]: (s) (uvwxyz); u[3]: (stxyz) (v) (w); v[2]: (stuxyz) (w); x[3]: (stuvw) (y) (z); y[2]: (stuvwx) (z). Six independent modulations are necessary for the elasticities of all the enzymes to be determined.

4.4. GENERALIZATIONS

Observations and analyses of how many and which enzymes have to be modulated for determining the elasticities of enzyme e can be summarized as follows:

(1) The minimum number $M_{\min(e)}$ of modulations required is the number of metabolites that affect the rate law of enzyme e, i.e. the number of non-zero elasticities for the enzyme. In a simple chain, $M_{\min(e)}$ is 2 for all e except for those at the ends of the chain.
(2) For the simple chain, modulation of any one enzyme downstream of e and any one enzyme upstream of e allow determination of the elasticites of e. This same rule holds for a pathway with a branch point, if "upstream" and "downstream" are understood to include also all enzyme in the two branches in which e is not located.
(3) If the chain has one regulatory loop (either feedback or feedforward), and e happens to be the target enzyme of the loop, three modulations are required, one located within the loop, one upstream and downstream of the loop; if e is not the target enzyme, one of the two modulations has to be located upstream and the other downstream from e; if e is located within the loop (but not adjacent to the effector), another modulation located downstream from the loop can substitute for the upstream modulation [compare w[2]: (s t u v) (x) (y z) for Fig. 2].

This list is by no means comprehensive. It is rather to indicate that general yet simple rules can be formulated for the identification of enzymes that have to be modulated for the determination of elasticities. Together with the recently developed procedure to actually carry out such measurements (Giersch, 1995; Hofmeyr *et al*., 1993), the scene is set for a more rapid pace of experimental progress in metabolic control analysis. The two approaches are not mutually exclusive, and each has advantages over the other. Modulation of all enzymes (Hofmeyr *et al*., 1993) avoids making any assumptions about the existence of zero elasticities, but it implies a considerable amount of experimental effort, which may often be superfluous, as information about the regulatory structure of a pathway may make it easy to guess which elasticities are likely to be zero. In such circumstances modulating only the minimum number of enzymes, as discussed in the present paper, is likely to yield as much information as modulating all of them.

5. Mathematical Background

Here we give a brief account of the mathematics by means of which the above examples have been analysed. In the Appendix, an example is given how the combinations of independent modulations can be listed using the software package Mathematica (Wolfram Research, Champaign, IL, U.S.A.).

To allow coherent representation of vectors and matrices the conventional symbolism using indices will be used in this section instead of the simpler system used elsewhere in this paper. The metabolic pathway is assumed to have n enzymes e_i, $i = 1 \cdots n$, and m metabolites x_j, $j = 1 \cdots m$. When the concentration of a metabolite x_j is regarded as a system variable it is symbolized S_j, but when it is considered a local variable, as for example in the definition of an elasticity for an isolated enzyme, it is symbolized x_j. We find it conceptually useful to preserve this distinction, but readers who do not share this view can treat the symbols S_j and x_j as synonymous. The steady-state flux J^i through enzyme e_i can be written as a function of the rate v_i of the isolated enzyme and of the steady-state metabolite concentrations S_j (which themselves depend on parameter values $p_1 \cdots p_n$) in the following way:

$$J^i = J^i[v_i, S_1(p_1 \cdots p_n), \ldots S_m(p_1 \cdots p_n)]. \qquad (1)$$

We assume that for any enzyme e_i there exists a specific modulator, i.e. that there is a parameter p_i such that $\partial v_i/\partial p_i \neq 0$ but $\partial v_i/\partial p_k = 0$ if $k \neq i$. It should be noted that the value of $\partial v_i/\partial p_i$ will usually not be known, and does not need to be (apart from knowing that it does not vanish). The fact that it is not necessary to know the percentage modulation in v_i caused by a given change in p_i is the major advantage of this approach (Hofmeyr *et al*., 1993; Giersch, 1994). Differentiating eqn (1) with respect to the p_i, we obtain the following matrix equation:

$$\begin{bmatrix} \frac{\partial J^1}{\partial p_1} & \cdots & \frac{\partial J^1}{\partial p_n} \\ \vdots & & \vdots \\ \frac{\partial J^n}{\partial p_1} & \cdots & \frac{\partial J^n}{\partial p_n} \end{bmatrix} = \begin{bmatrix} \frac{\partial v_1}{\partial p_1} & & 0 \\ & \ddots & \\ 0 & & \frac{\partial v_n}{\partial p_n} \end{bmatrix}$$

$$+ \begin{bmatrix} \frac{\partial v_1}{\partial x_1} & \cdots & \frac{\partial v_1}{\partial x_m} \\ \vdots & & \vdots \\ \frac{\partial v_n}{\partial x_1} & \cdots & \frac{\partial v_n}{\partial x_m} \end{bmatrix} \begin{bmatrix} \frac{\partial S_1}{\partial p_1} & \cdots & \frac{\partial S_1}{\partial p_n} \\ \vdots & & \vdots \\ \frac{\partial S_m}{\partial p_1} & \cdots & \frac{\partial S_m}{\partial p_n} \end{bmatrix}. \qquad (2)$$

Subtracting the diagonal matrix Diag($\partial v_i/\partial p_i$) from both sides of eqn (2), replacing the unknowns ($\partial J^i/\partial p_i - \partial v_i/\partial p_i$) on the principal diagonal of the resulting matrix by "?", and transposing the matrices on both sides, we get the following system of equations:

$$\begin{bmatrix} \frac{\partial S_1}{\partial p_1} & \cdots & \frac{\partial S_m}{\partial p_1} \\ \vdots & & \vdots \\ \frac{\partial S_1}{\partial p_n} & \cdots & \frac{\partial S_m}{\partial p_n} \end{bmatrix} \begin{bmatrix} \frac{\partial v_1}{\partial x_1} & \cdots & \frac{\partial v_n}{\partial x_1} \\ \vdots & & \vdots \\ \frac{\partial v_1}{\partial x_m} & \cdots & \frac{\partial v_n}{\partial x_m} \end{bmatrix}$$

$$= \begin{bmatrix} ? & \frac{\partial J^2}{\partial p_1} & \cdots & \frac{\partial J^{n-1}}{\partial p_1} & \frac{\partial J^n}{\partial p_1} \\ \frac{\partial J^1}{\partial p_2} & ? & \cdots & \frac{\partial J^{n-1}}{\partial p_2} & \frac{\partial J^n}{\partial p_2} \\ \vdots & \vdots & \ddots & \vdots & \vdots \\ \frac{\partial J^1}{\partial p_{n-1}} & \frac{\partial J^2}{\partial p_{n-1}} & \cdots & ? & \frac{\partial J^n}{\partial p_{n-1}} \\ \frac{\partial J^1}{\partial p_n} & \frac{\partial J^2}{\partial p_n} & \cdots & \frac{\partial J^{n-1}}{\partial p_n} & ? \end{bmatrix} \tag{3a}$$

or in short

$$\left(\frac{\partial \mathbf{S}}{\partial \mathbf{p}}\right)^{\mathrm{T}} \left(\frac{\partial \mathbf{v}}{\partial \mathbf{x}}\right)^{\mathrm{T}} = \left[{}^{?}\left(\frac{\partial \mathbf{J}}{\partial \mathbf{p}}\right)_{?}\right]^{\mathrm{T}}. \tag{3b}$$

This is a linear system for the unknown elasticities $\partial v_i/\partial x_j$. To solve it for the elasticities of e_r [i.e. to calculate the column vector $(\partial v_r/\partial x_1 \cdots \partial v_r/\partial x_m)^{\mathrm{T}}$], row r has to be omitted from the matrix $(\partial \mathbf{S}/\partial \mathbf{p})^{\mathrm{T}}$, as the entry (r, r) on the r.h.s. of eqn (3) is unknown. We then obtain a system of $n-1$ equations for the m unknown elasticities of v_r. As there are m metabolites and a greater number, n, of enzymes it follows that there are always at least as many equations as there are unknowns. Moreover, often only two or three of the elasticities in $(\partial v_r/\partial x_1 \cdots \partial v_r/\partial x_m)^{\mathrm{T}}$ are expected to be different from zero (see examples of Figs 1–6), and so it is sufficient to select, for any such vector $(\partial v_r/\partial x_1 \cdots \partial v_r/\partial x_m)^{\mathrm{T}}$, two or three rows of the matrix $(\partial \mathbf{S}/\partial \mathbf{p})^{\mathrm{T}}$ to get as many equations as there are non-zero unknowns in $(\partial v_r/\partial x_1 \ldots \partial v_r/\partial x_m)^{\mathrm{T}}$. While selecting these it is essential to make sure that they are linearly independent, or, in technical terms, that the 2- or 3-minors of the matrix $(\partial \mathbf{S}/\partial \mathbf{p})^{\mathrm{T}}$ are different from zero.

One can determine whether a minor of matrix $(\partial \mathbf{S}/\partial \mathbf{p})^{\mathrm{T}}$ is zero from the fact that the matrix $\mathbf{Q} = \{[\mathbf{N}(\partial \mathbf{v}/\partial \mathbf{x})]^{-1}\mathbf{N}\}^{\mathrm{T}}$ has the same structure of non-vanishing minors as the matrix $(\partial \mathbf{S}/\partial \mathbf{p})^{\mathrm{T}}$, where $\mathbf{N}$ is the stoichiometric matrix. Thus, a minor of $(\partial \mathbf{S}/\partial \mathbf{p})^{\mathrm{T}}$ is exactly zero when the corresponding minor in $\mathbf{Q}$ is zero. This allows the minors of $(\partial \mathbf{S}/\partial \mathbf{p})^{\mathrm{T}}$ to be calculated explicitly once the stoichiometric matrix $\mathbf{N}$ and the elasticity matrix $\partial \mathbf{v}/\partial \mathbf{x}$ are known.

The equivalence of the two matrices in relation to vanishing minors follows from differentiating the steady-state condition for a metabolic pathway (see Giersch, 1988) with respect to parameters p_i:

$$\mathbf{N}\left(\frac{\partial \mathbf{v}}{\partial \mathbf{x}}\right)\left(\frac{\partial \mathbf{S}}{\partial \mathbf{p}}\right) + \mathbf{N}\,\mathrm{Diag}\left(\frac{\partial \mathbf{v}}{\partial \mathbf{p}}\right) = \mathbf{0} \tag{4}$$

and hence

$$\left(\frac{\partial \mathbf{S}}{\partial \mathbf{p}}\right) = -\left[\mathbf{N}\left(\frac{\partial \mathbf{v}}{\partial \mathbf{x}}\right)\right]^{-1} \mathbf{N}\,\mathrm{Diag}\left(\frac{\partial \mathbf{v}}{\partial \mathbf{p}}\right). \tag{5}$$

As multiplying a matrix by a diagonal matrix does not change the pattern of vanishing minors it follows that $\partial \mathbf{S}/\partial \mathbf{p}$ and $[\mathbf{N}(\partial \mathbf{v}/\partial \mathbf{x})]^{-1}\mathbf{N}$ have identical minor structures.

Combinations of linear independent rows of $(\partial \mathbf{S}/\partial \mathbf{p})^{\mathrm{T}}$ that allow the determination of the unknown elasticities $(\partial v_r/\partial x_1 \cdots \partial v_r/\partial x_m)^{\mathrm{T}}$ can then be found as follows: delete from the matrix $\{[\mathbf{N}(\partial \mathbf{v}/\partial \mathbf{x})]^{-1}\mathbf{N}\}^{\mathrm{T}}$ row r and all columns in which the entries in $(\partial v_r/\partial x_1 \cdots \partial v_r/\partial x_m)^{\mathrm{T}}$ are zero. This generates a matrix $\mathbf{Q}^r$ with $n-1$ rows (corresponding to the enzymes) and q columns, where q is usually two or three, corresponding to the metabolites that are "seen" by v_i. Check which of the q-minors of the matrix $\mathbf{Q}^r$ are different from zero. Combinations of enzymes appearing in non-vanishing minors are those for which modulations are independent.

If the entries to matrix $\partial \mathbf{v}/\partial \mathbf{x}$ are written in symbolic form (as $\partial u/\partial A$, $\partial u/\partial B$, etc.) calculation of the minor structure can produce output of enormous length. This can be avoided by replacing all non-zero entries in $\partial \mathbf{v}/\partial \mathbf{x}$ with random numbers: the probability is very high that the minors calculated numerically in this way will be zero only if the corresponding minors in the algebraic expression are zero, especially if the calculation is repeated with a different set of random numbers. As an illustration of this numerical procedure for solving a structural problem, a short Mathematica code producing a list of the minors for the pathway of Fig. 4(a) is given in the Appendix.

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APPENDIX

The following Mathematica code produces lists of the minors occurring in course of the calculation of the elasticities for the pathway of Fig. 4(a).

```
stoich={{1, -1,  0,  0,  0,  0},
        {0,  1, -1,  0,  0,  0},
        {0,  0,  1, -1,  0,  0},
        {0,  0,  0,  1, -1,  0},
        {0,  0,  0,  0,  1, -1}};

epsilon={{Random[ ], Random[ ],     0,          0,          0,          0},
         {    0,     Random[ ], Random[ ],     0,          0,          0},
         {Random[ ],     0,     Random[ ], Random[ ],     0,          0},
         {    0,         0,         0,     Random[ ], Random[ ],     0},
         {    0,         0,     Random[ ],     0,     Random[ ], Random[ ]}};
```

`stoich` is the stoichiometric matrix **N**, and `epsilon` is the transpose $(\partial \mathbf{v}/\partial \mathbf{x})^{T}$ of the elasticity matrix $\partial \mathbf{v}/\partial \mathbf{x}$ with the non-zero entries replaced by random numbers. Matrix **Q** is represented in the Mathematica code by `ders`, and is then calculated as follows:

```
pl=stoich . Transpose[epsilon];
invpl=Det[p1] Inverse[p1];
ders=Transpose[invpl.stoich];
```

The submatrices $\mathbf{Q}^{r}$, r = u · · · y of **Q** allowing calculation of the elasticities of enzyme r are obtained by omitting row r from **Q** and keeping only those columns of **Q** for which the entries in column r of `epsilon` are non-zero:

```
mu=ders[[{2, 3, 4, 5, 6}, {1, 3}]];
mv=ders[[{1, 3, 4, 5, 6}, {1, 2}]];
mw=ders[[{1, 2, 3, 4, 5, 6}, {2, 3, 5}]];
mx=ders[[{1, 2, 3, 5, 6}, {3, 4}]];
my=ders[[{1, 2, 3, 4, 6}, {4, 5}]];
```

The following input lines list the 2-minors or 3-minors in alphabetical order:

```
Minors [mu, 2];
Minors [mv, 2];
Minors [mw, 3];
Minors [mx, 2];
Minors [my, 2]
{{0.}, {0.}, {0.}, {0.052931}, {0.}, {0.}, {−0.10863}, {0.}, {0.0666483},
{−0.0283475}}
```

From the output (which is reproduced here only for the last input line giving the minors for pairs of enzymes from the list (u, v, w, x, z) in alphabetical order) one can see immediately which minors are different from zero. The above code can be extended to print lists of combinations of the enzymes for which modulations allow determination of the elasticities.