Structural and Organizational Aspects
of Metabolic Regulation, pages 273–289

CONTROL ANALYSIS OF ORGANISED MULTIENZYME SYSTEMS[1]

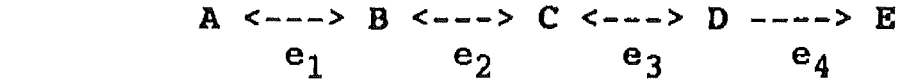
Douglas B. Kell[2] and Hans V. Westerhoff[3]

[2]Dept of Biological Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DA, U.K.
[3]Division of Molecular Biology, Nederlands Kanker Instituut, H5, Plesmanlaan 121, 1066CX AMSTERDAM, The Netherlands.

ABSTRACT We review the metabolic control analysis (MCA) developed by Kacser, Burns, Heinrich, Rapoport and others, and illustrate how it may be used as a rigorous means of distinguishing systems exhibiting 'pool' behaviour from those exhibiting 'channelling'.

INTRODUCTION

Much of metabolism *in vivo* may be organised such that intermediary metabolites are passed from enzyme to enzyme without becoming solvated by the bulk of the aqueous cytoplasm, and do not exhibit 'pool' behaviour. The evidence for this type of view (variously referred to as 'micro-compartmentation', 'channelling' or 'localised coupling') is both structural and functional, and since it has recently been reviewed *in extenso* (e.g. [1-4]) it is not necessary to reiterate it here. Such a view has it that for a pathway such as:



[1]This work has been supported by the SERC Biotechnology Directorate, UK, and the NWO, The Netherlands.

any molecule of (say) C produced by a molecule of enzyme e_2 in a membrane-bounded compartment is not freely and equally available to each molecule of e_3. Given that the diffusion coefficients of small molecules *in vivo* are moderately high (ca. 10^{-6} $cm^2.s^{-1}$ [5]), this would generally imply that the substrate for enzyme e_3 is not C but an e_2C complex.

The metabolic control analysis (MCA) devised by Kacser, Burns, Heinrich and Rapoport in the early 1970s, and more recently extended by others (see later), provides a formalism for the quantitative description of the control of metabolism under steady-state conditions, and relates the 'local' kinetic properties of enzymes to their 'global' properties such as their contribution to the control of variables such as fluxes and intermediary metabolite concentrations. In its original form, it treated metabolism as though it was organised only according to the 'pool' concept. However, we [6] and others [7-9] have recently described particular ways in which it may be used to establish the existence and significance of 'metabolic channelling' (and see also Welch, this volume).

Thus the principal purposes of this article are (i) to review in outline the principles and practice of the metabolic control analysis, and (ii) to describe some of the ways in which it may be applied to the analysis of systems exhibiting metabolic channelling.

METABOLIC CONTROL ANALYSIS - A GUIDE FOR THE PERPLEXED [10-22].

Control Coefficients

Flux-control coefficients. The first problem in the study and description of metabolic control is to determine what we mean by a pathway. The operational definition used in the MCA is that a pathway is a system that consists of a flux from a starting substrate at an effectively fixed concentration to a product at an effectively fixed concentration. 'Effectively fixed' here means

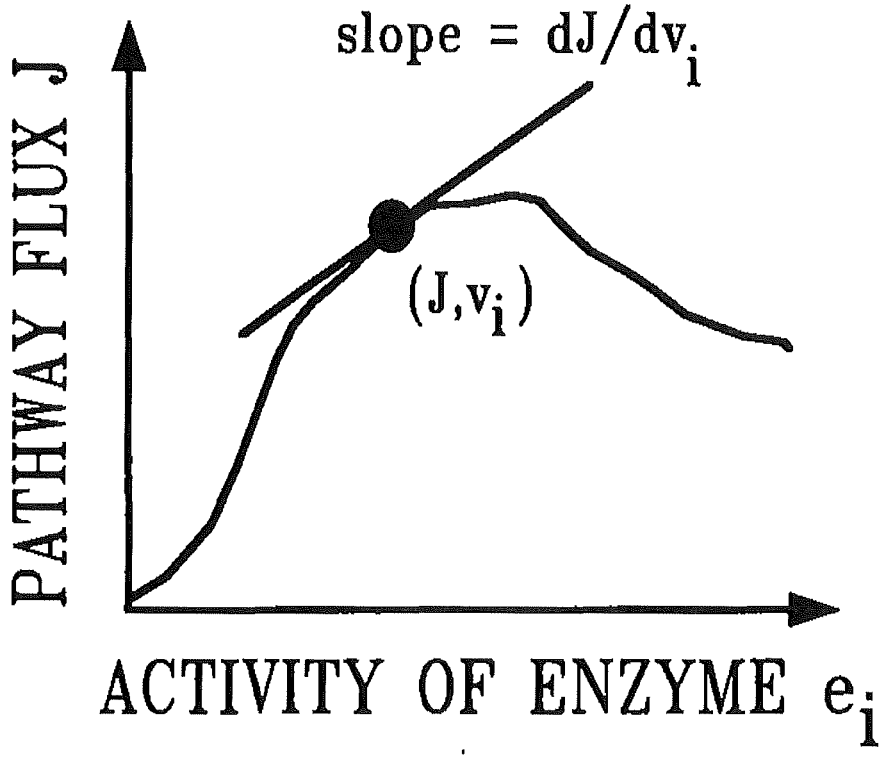


Fig 1. The relationship between a metabolic flux and an enzyme activity may be strongly nonlinear. To define the flux-control coefficient we must make small changes in the activity of the enzyme of interest and measure the consequent changes in pathway flux. The flux-control coefficient is the ratio of these fractional changes.

either that the concentration in question is kept fixed by some external means (e.g. a buffer or a large volume), or that the concentration varies in a range where the variation does not affect the activity of any of the enzymes in the pathway (e.g. if the substrate concentration is far above the K_m and the product concentration far below the K_p). This functional isolation of the pathway of interest from the rest of the cellular metabolism is based upon the differential relaxation times of different parts of metabolism, i.e. the temporal existence of a steady state that is not affected by other responses of the system such as the induction of relevant gene products.

In our prototypical pathway (A ---> E) above, it is traditional to ask questions such as "which enzyme is rate-limiting"? The metabolic control analysis, which was introduced by Kacser and Burns [23] and Heinrich and Rapoport [24], shows that the contribution of an individual enzyme to the control of flux through a pathway is a systemic property, which tends to be rather subtle. Now, whilst we know that removing all of the enzyme in a pathway will reduce the flux to zero, this only tells us that the enzyme is in the pathway of interest. To obtain a meaningful analysis (Fig 1), we must determine the change in flux caused by a very small (strictly speaking, infinitesimal) change in enzyme activity (V_{max}). (In non-organised systems enzyme concentration may be substituted for enzyme activity.) To obtain a dimensionless number, we use the fractional change in enzyme activity and in flux. Thus, using the new, unified terminology [25], one defines a **flux-control coefficient** $C^J_{e_i}$ as $(dJ/J)/(dv_i/v_i)_{SS}$ = $(d \ln J / d \ln v_i)_{SS}$, where v_i is the activity of enzyme e_i, and the subscript SS (steady-state) implies that the comparison is made after the system has relaxed to its steady state(s). Thus the flux-control coefficient equals the slope of a log-log plot of J vs v_i at the concentration (activity) of e_i prevailing.

The importance of the flux-control coefficient lies in the fact that, as shown by the **flux-control summation theorem**, and for systems exhibiting pool behaviour, the sum of the flux-control coefficients of the enzymes in (or acting upon) a pathway = 1. This means that if we determine a flux-control coefficient for an enzyme of say 0.2, we know that we must look elsewhere for the rest of the flux-control. It may be noted that if one considers branched pathways, the enzymes in the branch other than that containing the flux of interest ('reference flux') will tend to have negative flux-control coefficients (increasing their activity will decrease the flux of interest), so that the sum of the flux-control coefficients of the enzymes in the pathway of interest will tend to exceed 1. It should also be mentioned that the MCA is exact only because it

considers small changes around the steady state. Larger departures are considered in theories such as the Biochemical Systems Theory developed by Savageau and his colleagues [26-29], which have the benefit of a larger domain of applicability in 'control space' at the cost of some loss in precision.

Other Control Coefficients. We may also define control coefficients for the control of flux by the external (starting) substrate concentration (C^J_S) and by external modulators such as inhibitors (C^J_I), or for the control of intermediary metabolite concentrations ([X]) by enzyme activities (concentrations) ($C^X_{v_i}$). The latter are known as metabolite concentration-control coefficients, and have a summation theorem equal to zero [30]. Each of these coefficients are defined in a similar way to the flux-control coefficients as (d ln superscript / d ln subscript). Control coefficients of enzymes on the transient time of a pathway have also been defined [30].

Parameters and variables

The MCA lays great stress on the distinction between parameters and variables. **Parameters** are those factors which are set by the experimenter (typically temperature, pH, starting substrate concentrations, etc) or by the system itself (typically K_m, K_i and V_{max} values), and are unchanging during the course of an experiment. As it stands, therefore, MCA does not consider changes in enzyme concentrations caused for instance by the induction or repression of genes, although extensions to cover these cases exist [31]. **Variables** are those factors which attain a constant value only when the system attains a stable steady state. The most important variables are the flux J and the concentrations of intermediary metabolites. It should be stressed that variables cannot control fluxes, so that it is quite incorrect to ascribe a control of a flux to a low concentration (relative to a K_m) of an intermediary metabolite.

Elasticity coefficients

Of course, enzyme activities *in vivo* do depend upon the concentrations of their substrates (and of other effectors), and the MCA describes these as **elasticity coefficients** or **elasticities**. These are defined in a form mathematically similar to the control coefficients; thus, the elasticity of enzyme e_i towards (the concentration of) substrate X is $\varepsilon^{e_i}_X$ = d ln v_i / d ln X, i.e. the fractional change in enzyme turnover number caused by a fractional change in substrate concentration. The derivatives here are *partial* derivatives since the conditions are constrained such that all other parameters *and* variables (here such as the concentrations of the product and any allosteric modifiers of the enzyme) are held constant at their *in vivo* (steady state) values.

The connectivity theorems.

The behaviour of the metabolic system of interest does depend upon that of its constituent parts, and the MCA formalises this in terms of the so-called **flux-control** and **concentration-control connectivity theorems**. The easiest way to think about the flux-control connectivity theorem is to imagine adding a non-competititive inhibitor to a steady-state system such as the A ----> E pathway considered earlier. If the inhibitor is a specific inhibitor of enzyme e_3, the first effect will tend to be a build-up of intermediate C. This will either cause enzyme e_3 to speed up (if [C] had been at or below the K_m of e_3) or will have no effect (if e_3 was already saturated with C). In the first case, e_3 would have a high elasticity (large change in turnover for small change in substrate concentration) but a low flux-control coefficient (little change in pathway flux for a significant change in enzyme concentration), whereas the second case would be the other way round. More generally, the flux-control connectivity theorem shows that the sum of the products of the flux-control coefficients of the enzymes in a pathway and their elasticities towards a given metabolite is zero. Other theorems

relate the metabolite concentration-control coefficients to the elasticities, and indeed a number of matrix methods have been devised which relate the control coefficients to the elasticities [32-37]. For linear pathways, it is possible to express the control coefficients in terms of the elasticities alone, whereas branched pathways require in addition a knowledge of the flux-ratio at the branches [33, 34, 36].

Finally, for a tight-binding and specific inhibitor, the flux-control coefficient of the inhibitor = the flux-control coefficient of the target enzyme times the elasticity of the target enzyme towards the inhibitor, such that for a 'perfect' tight-binding and non-competitive inhibitor, the flux-control coefficient = the ratio of the initial slopes of normalised flux and normalised enzyme activity when plotted against the inhibitor concentration [6, 10, 11, 13, 14, 20].

Activities or concentrations?

Both in the thermodynamic sense, and as regards enzymic properties, we must decide whether we should use activities or concentrations in our description of metabolic control. In the first (thermodynamic) case, we use activities, which we equate with free concentrations. Though not experimentally obtainable by methods such as perchloric acid extraction (which of course gives the values for 'bound' plus 'free' metabolites) they may be derived by means such as NMR analysis. As regards the enzymic properties, our choice is open. The Berlin group [30] defines the control coefficients and elasticities in terms of the 'local (in situ) activity' of the enzymes, and on this basis the control analysis is valid for 'channelled' as well as 'pool' systems. The problem with this approach is that it is virtually impossible to measure the 'local activity' in the case of channelled systems. The alternative approach adopted by the Edinburgh group [9] is to use the more-easily-measured enzyme concentrations and to allow for enzyme-enzyme interactions and channelling by means of another matrix (the so-

called pi-elasticities) which relates the *in situ* activities to the concentrations via an algebraic term. Whilst this approach is not without its difficulties (see Welch, this volume), it is perhaps easier to gain a physical picture of what is going on. To see why, we must discuss some of the ways in which one may seek to measure flux-control coefficients (for the measurement of elasticities, readers are referred e.g. to [20]).

Measurement of flux-control coefficients

The measurement or estimation of flux-control coefficients follows directly from their definition: one modulates the concentration or activity of an enzyme and measures the consequent change in flux under steady-state conditions in which no other parameters have changed. Methods for doing this, with selected examples, include (a) variation of enzyme concentrations in systems reconstituted *in vitro* [18, 38], (b) inhibitor titrations [10, 39, 40], (c) variation of enzyme concentration by expression in diploid organisms [41, 42] and (d) modulation of enzyme concentration by recombinant DNA methods in which the expression may be controlled by using a promoter of variable strength [43] or by other molecular cloning methods [44, 45]. Method (b) requires that the specificity of the inhibitors used is known, and preferably absolute, whilst the last two methods require that pleiotropic effects are absent.

More importantly, each of these methods suffers from the problem that as the flux-control coefficients become small, as they will indeed tend to do for long pathways, they becomes increasingly difficult to distinguish from zero, and in fact, with these approaches, values less than approximately 0.1 may be subject to large relative errors. (Note that the absolute errors are far lower, and to find say a flux-control coefficient of 0.1 or less is very meaningful.) In say an inhibitor titration, the accuracy also depends upon how far one may inhibit the flux before the flux-control coefficient itself changes significantly (i.e. the curve bends round). In

some pathways, such as that described in Fig 5 of reference [20], this may be a long way, whereas in other cases (e.g. [46]) the distribution of control depends strongly on the absolute flux. The biological significance of these very interesting differences is not yet understood. Statistical problems associated with the estimation of flux-control coefficients are discussed by Small [47].

Flux-control coefficients in supercomplexes. If one is trying to distinguish 'pool' from 'channelled' metabolism, a particularly interesting problem arises. To describe it we may imagine a 'perfect' channel, in which 'free' metabolites either do not exist or are not used (significantly) as substrates for 'their' enzymes due to unfavourable K_m values. In this case, the entire pathway and its intermediates behave as a 'supercomplex' such that inhibiting one of the enzymes present (by say 1%), using an 'irreversible, all-or-none' type of inhibitor, will inhibit the flux in direct proportion so that the enzyme would have a flux-control coefficient of 1. (This would not be so for an inhibitor that simply reduces the probability of an individual reaction.) This would of course be true for each of the enzymes in the supercomplex, so that the flux-control summation theorem would appear to be violated when judged by these means [6, 48], since if the supercomplex contains n enzymes, the sum of the apparent flux-control coefficients would be n. By contrast, if one modulated the concentration of enzyme present in the system by adding enzyme (either directly or by cloning), the enzyme added would not be able to participate in supercomplex formation, so that adding enzyme would not increase the flux and the flux-control coefficient would be zero! For instance, in the experiments of Heinisch [44], who increased the concentration of the phosphofructokinase (PFK) enzymes by cloning the 2 relevant structural genes, the data suggested that PFK had a rather low flux-control coefficient (although the data, in terms of the constancy of the ethanologenic flux, are not good enough to exclude a value below 0.1). If one were to carry out similar experiments for the rest of glycolysis (more than 13 enzymes), obtaining

similar data in each case, one might conclude one of the following: either the system exhibits pool behaviour, the distribution of control is rather homogeneous and the flux-control coefficients are too small to measure reliably, or the system operates as a supercomplex (a proposal for which much evidence in fact exists in a variety of systems [49-51]). Thus, as also mentioned by Brindle [45], the only way to distinguish these possibilities by the approach described is if cloning both 'up' and 'down' in enzyme concentration is performed.

Double-inhibitor titrations.

It is often not possible to determine the flux-control coefficients of all the enzymes in the pathway. In such cases one may apply the so-called double-inhibitor titration method. The principle of this method is that, in general, inhibition of one of the enzymes in a linear, non-channelled pathway, makes that enzyme less flux-controlling (and hence, by virtue of the flux-control summation theorem, the other enzymes more flux-controlling). In other words, inhibition of one of the enzymes should reduce the effect on the pathway flux of a given small amount of an inhibitor of any of the other enzymes.

In terms of the MCA, one is here determining the sign of a double cross derivative of the flux with respect to two enzyme concentrations. Just as for the normal flux-control coefficients, the "double" cross control coefficients can be expressed in terms of elasticity coefficients and (if there are branches) flux ratios at the branches [6]. These expressions for the double control coefficients are strongly suggestive of the sign of the coefficients, at least when the enzymes have the common properties that their reaction rates increase with [S], decrease with [P], and where the "turnover elasticity coefficients" (corresponding to the product of the elasticity coefficient and turnover number) are fairly constant. We derived these relationships for a three-enzyme, branched pathway, and demonstrated that the cross coefficients should be

negative, i.e. that inhibitors of two different enzymes in a pathway leading to a certain output flux should have antisynergistic effects on that flux, in line with the general principle stated above [6].

Advantages of this method include the facts that (a) one generally does not have quantitatively to evaluate the flux-control coefficients (which may require detailed knowledge of the properties of the inhibitor [10]), and (b) one needs only two inhibitors, even if the (segment of interest of the) pathway contains many more enzymes. Disadvantages include the fact that the inhibitors must be specific for their targets and that the principle is not universally valid: there are properties of enzymes that may give the same behaviour even in a non-channelled pathway. An extensive analysis has been given elsewhere [6,7].

The method has been primarily applied to the question of whether proton-mediated free-energy transduction in oxidative and photophosphorylation involves channelled fluxes of energy quanta [reviews: 52, 53], and has recently been analysed in extenso [6,7].

Failure of connectivity

As discussed above, connectivity theorems relate control coefficients to enzyme properties (i.e. the elasticities). Thus, in a simple, non-channelled, linear pathway, the ratio of the flux-control coeficients of two adjacent enzymes equals minus the inverse ratio of the elasticities of these enzymes to their common metabolite. In a completely channelled pathway, the ratio of the flux-control coefficients (as measured by inhibition with an all-or-none inhibitor) equals 1. This would not in general be equal to the minus the ratio of the elasticity coefficients. Whilst we recognise the difficulties of measuring elasticities under these conditions, this method of detecting channelling does not appear to have been implemented.

CONCLUDING REMARKS

Metabolic control analyses of metabolic pathways lead to quantitative insights into the degrees to which enzymes control concentrations and fluxes. If a metabolic pathway is not channelled, the (properly measured) flux-control coefficients must add up to 1.

In addition, metabolic control theories may be used to determine whether metabolism is channelled, since certain types of failure of the system to obey the theorems of metabolic control analysis constitute good indications for channelling. The following failures are particularly useful: (i) failure of the flux-control coefficients to add up to 1; (ii) dependence of the flux-control coefficient on the method used to vary the enzyme activity (up vs. down); (iii) failure of two inhibitors to act antisynergistically; (iv) failure of the connectivity theorems.

ACKNOWLEDGMENTS

We thank Rick Welch for many enjoyable and stimulating discussions.

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