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# Quantitative approaches to the analysis of the control and regulation of microbial metabolism

Hans V. Westerhoff[1,2], Wally van Heeswijk[1,2], Daniel Kahn[1] and Douglas B. Kell[3]
[1] *Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands;* [2] *E.C. Slater Institute for Biochemical Research, University of Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands*
[3] *Department of Biological Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DA, UK*

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**Abstract**

Recently, a number of novel ways of considering the control, regulation and thermodynamics of microbial physiology have been developed and applied. We here present an overview of the new concepts involved, of their limitations and of the most recent attempts to deal with those limitations. We conclude that there no longer exist reasons of principle for vagueness in discussions of the control of microbial physiology and energetics. Further, the novel conceptual methods serve to remove part of the discordance between holistic and reductionistic views of microbial physiology.

## 1. Introduction

In a microbial cell of a few cubic microns, thousands of processes occur simultaneously at rather appreciable rates. For the fitness of the cell it is mandatory that the rates of these processes are well adjusted to each other. Whilst some of this adjustment occurs 'automatically' through mass-action effects, much involves more sophisticated control mechanisms. Biochemists and molecular biologists may study control at the level of single processes, whilst cell physiologists may consider control and regulation at the integrated level of entire cells. In general, however, the sheer complexity of metabolism has precluded the establishment of strict relationships between the molecular and the cellular level. In recent years, however, concepts and methodologies have been developed that serve to relate the two levels. Because they have to deal with complexity, many of these methods implement mathematics, yet they have brought concepts that can also be grasped and employed more intuitively. Whilst giving some insight into the quantitative power of some of these methods, this overview will lean towards conveying some of the conceptual advances which they represent.

We shall begin by reviewing some of the *concepts* of a method rationally to analyze the control of metabolic fluxes in metabolic pathways, i.e., Metabolic Control Analysis (MCA; a review of applications of MCA to microbial physiology will be found elsewhere in this volume; Van Dam & Jansen 1991). Subsequently, we shall outline nonconventional aspects of MCA that pertain to chemostats. Next we shall point at two limitations inherent to conventional MCA, i.e., its emphasis on metabolism in cells of constant composition with immutable enzyme activities, and its limitation to small changes. Subsequently, we shall discuss some recent advances that lift these limitations. One is a recent extension of MCA that makes use of the fact that cellular physiology tends to be organized in

terms of recognizable chunks ('modules') that each carry out their own task and regulate each other. We shall elaborate this method in some detail for the particular case of ammonia assimilation in *E. coli*, as it is regulated through a covalent modification cascade affecting the activity of glutamine synthetase. Finally, we shall discuss analysis methods that may be used whenever changes are not small, such as Mosaic Non Equilibrium Thermodynamics and Biochemical Systems Theory.

## 2. Metabolic control analysis and microbial physiology: the concepts

The metabolic control analysis (MCA) devised by Kacser, Burns, Heinrich, Rapoport, and Rapoport in the early 1970s, and more recently extended by others (see later), provides both a strategy and a formalism for the quantitative description of the control of metabolism under (mainly) steady-state conditions. It relates the 'local' kinetic properties of enzymes to their 'global' properties like their contribution to the control of variables such as fluxes and intermediary metabolite concentrations. From this point of view, it constitutes in principle an ideal formalism for attacking the problems on which this Special Issue is focussed, viz. Quantitative Aspects of Microbial Metabolism. To this end, we begin by providing a short review of the salient features of MCA.

MCA has been reviewed several times in recent years (e.g. Groen et al. 1982; Kell & Westerhoff 1986 a, b; Brand & Murphy 1987; Kacser & Porteous 1987; Westerhoff & van Dam 1987; Kell et al. 1989; Kell & Westerhoff 1990; Cornish-Bowden & Cárdenas 1990; Van Dam & Jansen 1991). Its chief distinction, from our point of view, is that it can relate the properties of *individual* components of a system (e.g. 'external' metabolites, or enzymes in a metabolic pathway) to their global behavior in contributing to the control of a metabolic flux.

*Flux-control coefficients*

We first define 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 starting substrates at fixed concentrations (S in Fig. 1) to products that are also maintained at constant concentrations (P in Fig. 1). Alternatively, concentrations of substrates and products are such that changes in them do not affect enzyme activities in the pathway (because of saturation). The pathway is to include the production and utilization of all (generally allosteric) effector molecules ('internal effectors') acting on the pathway. This functional isolation of the pathway of interest from the rest of the cellular metabolism is based upon the fact that different parts of metabolism are or may be isolated kinetically from each other. In other words, in conventional MCA, we deal with metabolic steady states that are not affected by other responses of the system such as the induction of relevant gene products.

When studying a typical metabolic pathway, not least in terms of maximizing fluxes of biotechnological interest, it is traditional to ask questions such as 'which enzyme is rate-limiting'? The metabolic control analysis shows that the contribution of an individual enzyme to the control of flux through a pathway is both a *systemic* property and can be expressed in quantitative terms. Now we know, of course, that removing all of the enzyme in a pathway will reduce the flux to zero; however, this only tells us that the enzyme is *in* the pathway of interest. To obtain a meaningful analysis, therefore, we must determine the change in flux caused by a small (strictly an infinitesimal) change in enzyme activity ($k_{cat}$ or $V_{max}$). To obtain a dimensionless number, we use the fractional change in enzyme activity and in flux. Thus, using the new, unified terminology (Burns et al. 1985), we define 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). J represents the steady-state flux through the system. Here it is to be understood that the change in enzyme activity $v_i$ is brought about by changing a parameter that affects that activity. The flux-control coefficient therefore equals the slope of a log-log plot of J *vs* $v_i$ at the concentration (activity) of $e_i$ prevailing.

Except for systems that exhibit 'channelling'

rather than pool behavior (see Welch & Keleti 1988; Kacser et al. 1990; Kell & Westerhoff 1990; Welch & Keleti 1990; Ovádi 1991), the sum of the flux-control coefficients of the enzymes in (or acting upon) a pathway equals 1. This relationship, known as the *flux-control summation theorem*, means that if we find that a particular enzyme has a flux-control coefficient of say 0.1, we know the rest of the flux control resides in other enzymes. It is also worth pointing out 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 (since increasing their activity will decrease the flux of interest); the sum of the flux-control coefficients of the enzymes in the branch of interest will therefore tend to exceed 1 (Kacser 1983).

*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) ($C^X_{e_i}$). The latter are known as metabolite concentration-control coefficients, and have a summation equal to zero. Each of these coefficients are defined in a similar way to the flux-control coefficients, i.e. as (d ln superscript / d ln subscript)$_{ss}$.

*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 and the starting or 'clamped' substrate concentrations) or by the system itself (typically in this case $K_m$, $K_i$ and $V_{max}$ values), *and which are unchanging during the course of an experiment.* As it stands, therefore (but see later), conventional MCA does not consider changes in enzyme concentrations caused for instance by the induction or repression of genes. 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. We would also stress that variables cannot control fluxes, so that it is quite inappropriate to ascribe a control of a flux to a low concentration (relative to a $K_m$) of an intermediary metabolite, say; a particular metabolite concentration adopts a low value in a steady state because of the properties of the enzymes producing and consuming it.

A. $S \rightleftharpoons Y \rightleftharpoons Z \rightleftharpoons P$

B. $S_0 \xrightarrow{\text{pump}} X \rightleftharpoons Y \rightleftharpoons Z \rightleftharpoons P$

*Fig. 1.* (A) a sample metabolic pathway. S and P are supposed to be present at effectively fixed concentrations, whereas the concentrations of Y and Z, as well as the fluxes can vary.
(B) Microbial culture in chemostat written so as to be amenable to metabolic control analysis; the first reaction is that of the pump introducing substrate, present in the feed at concentration $S_0$, into the chemostat. The substrate concentration in the chemostat is now a variable and referred to by X.

*Elasticity coefficients*

Of course, enzyme activities do depend upon the concentrations of their substrates (and of other effector molecules), and the MCA describes these interactions in terms of so-called elasticity coefficients or elasticities. These are defined in a form that is mathematically very similar to that of the definition of the control coefficients; thus, the elasticity of enzyme $e_i$ towards (the concentration of) substrate X is $\varepsilon^i_X = \partial \ln v_i / \partial \ln X$, i.e. the fractional change in enzyme turnover number caused by a fractional change in substrate concentration, subject to the important constraint that the change is carried out with all other parameters and variables held constant at their steady state values (Burns et al. 1985).

*The connectivity theorems*

Elasticities and control coefficients describe, in quantitative terms, respectively local and global properties of the metabolic system. The question

then arises as to how these may be related to each other, since of course the behaviour of the metabolic system of interest does depend upon that of its constituent parts. The MCA formalizes this in terms of the so-called flux-control and concentration-control connectivity theorems. Perhaps the easiest way to think about the flux-control connectivity theorem is to imagine adding a non-competitive inhibitor to a steady-state pathway consisting of a linear series of metabolites (A . . . --> . . . E) whose metabolism is catalyzed by a series of enzymes ($e_1$ to $e_4$) obeying reversible Michaelis-Menten kinetics. If the inhibitor is a specific inhibitor of enzyme $e_3$, the first effect will tend to be a build-up of its substrate (C). This will either cause enzyme $e_3$ to speed up (if [C] was originally somewhere near the $K_m$ of $e_3$) or will have no effect on the turnover of the enzyme (if $e_3$ was already saturated with respect to C). In the first case, from our definitions above, $e_3$ 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 effective enzyme concentration or activity), whereas in the second case the converse would be true. 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.

A number of approaches, some of which use matrix methods, have been devised which relate the control coefficients to the elasticities (e.g. Fell & Sauro 1985; Westerhoff & Kell 1987; Sauro et al. 1987; Reder 1988; Small et al. 1989; Cascante et al. 1989). For linear pathways, it is possible to express the control coefficients in terms of the elasticities alone, whereas branched pathways require, additionally, a knowledge of the flux-ratio at the branches (Westerhoff & Kell 1987; Sauro et al. 1987; Small & Fell 1989). For the pathway of Fig. 1A, the analysis is as follows (Westerhoff & Kell 1987; Sauro et al. 1987; Westerhoff & Van Dam 1987). First one writes the matrix **E**, which contains information both concerning the enzyme properties (elasticity coefficients, $\varepsilon$) and concerning the structure of the system (in the sense of where the flows flow):

$$\mathbf{E} = \begin{bmatrix} 1 & -\varepsilon_Y^1 & -\varepsilon_Z^1 \\ 1 & -\varepsilon_Y^2 & -\varepsilon_Z^2 \\ 1 & -\varepsilon_Y^3 & -\varepsilon_Z^3 \end{bmatrix}$$

Here the row of 1's reflects the fact that the example is that of a linear pathway. $\varepsilon_Y^1$ is the elasticity coefficient of the first reaction with respect to the concentration of metabolite Y, etc. Inversion of this matrix gives the matrix that gives all control coefficients:

$$\mathbf{C} = \begin{bmatrix} C_1^J & C_2^J & C_3^J \\ C_1^Y & C_2^Y & C_3^Y \\ C_1^Z & C_2^Z & C_3^Z \end{bmatrix} = \mathbf{E}^{-1}$$

$C_1^J$ quantifies the control exerted by enzyme 1 on the steady state pathway flux. $C_2^Z$ does this for the control exerted by enzyme 2 on the concentration of metabolite Z. This procedure can be extended to pathways of any complexity, where for the more complex cases, the method of Reder (1988) is the most systematic one (see also Holstein & Greenshaw 1991).

For a specific inhibitor, the flux-control coefficient of the inhibitor equals the flux-control coefficient of the target enzyme times the elasticity of the target enzyme towards the inhibitor. In other words, for a 'perfect' inhibitor, the flux-control coefficient equals the ratio of the initial slopes of normalized flux and normalized enzyme activity when plotted against the inhibitor concentration (Groen et al. 1982, Kell & Westerhoff 1986 a,b; Westerhoff & Kell 1988; Kell et al. 1989).

*Measurement of flux-control coefficients*

The measurement or estimation of flux-control coefficients follows in principle directly from their definition: one modulates the concentration or activity of an enzyme and measures the consequent change in flux, between steady-state conditions in which no other parameters have changed. Methods for doing this, with selected examples, include (see also van Dam & Jansen 1992): (a) titrations with

specific metabolic inhibitors (Groen et al. 1982; Walter et al. 1987; Cornish et al. 1988), (b) variation of enzyme concentration by variation of their expression in diploid organisms (Flint et al. 1981; Middleton & Kacser 1983), (c) modulation of enzyme concentration by recombinant DNA methods in which the expression may be controlled by using a promoter of variable strength such as the *tac* promoter (Walsh & Koshland 1985), or by other molecular cloning methods (Heinisch 1986; Schaaf et al. 1989), and (d) variation of enzyme concentrations in systems reconstituted *in vitro* (Torres et al. 1986). Method (a) requires that the specificity of the inhibitors used is known, and preferably absolute, whilst the molecular genetic methods require that pleiotropic effects are absent, at least for the systems studied using conventional MCA.

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 become increasingly difficult to distinguish from zero, and in fact, with these approaches, values less than approximately 0.1 are probably not very reliable quantitatively. 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 Kell et al. (1989), this may be a long way, whereas in other cases (e.g. Groen et al. 1982; Savageau & Voit 1982) 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 (1988) and Small & Fell (1990).

*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' (so-called 'static') channel (Keleti & Ovádi 1988), 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%) will inhibit the flux in direct proportion so that the enzyme would apparently have a flux-control coefficient of 1. If similar inhibitors were used for other enzymes in the complex, this would also be true for them, so that the flux-control summation theorem would appear to be violated when judged by these means (Kell & Westerhoff 1985, 1990; Westerhoff & Kell 1988); if the supercomplex contains n enzymes, the sum of the apparent flux-control coefficients would be n. In contrast, if one modulated the concentration of enzyme present in the system by *adding* enzyme (whether 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! Heinisch (1986) increased the concentration of the phosphofructokinase (PFK) enzymes by cloning the 2 relevant structural genes, and acquired data which suggested that PFK has a rather low flux-control coefficient (although the data, in terms of the constancy of the flux from glucose to ethanol, are probably not good enough to exclude a value below approximately 0.2). If one were to carry out similar experiments for the rest of the glycolytic system (more than 13 enzymes), one would probably obtain similar data in each case. Schaaf et al. (1989) extended this study to include 8 glycolytic enzymes, to similar effect. One might therefore conclude *either* that the system exhibits pool behaviour, the distribution of control is rather homogeneous and the flux-control coefficients are too small to measure reliably, *or* that the system operates as a supercomplex. These possibilities may be distinguished by cloning both 'up' and 'down' in enzyme concentration.

Van Dam and Jansen (1991) give a detailed overview of the application of MCA to understanding the control of microbial metabolism in a number of systems.

## 3. Metabolic control analysis, chemostats and bioreactors

In conventional control analysis, a pathway is de-

limited by substrates and products that are kept at constant concentrations (see above). In standard MCA, asking to what extent microbial growth is determined by the concentration of the growth-limiting substrate, takes the following form: One grows the cells at one, fixed substrate concentration and determines the growth rate. One then increases the substrate concentration by p%, maintains it at the new level whilst measuring the new steady-state growth rate (p being taken as small as consistent with accurate experimentation). The percentage change in growth rate divided by p is the coefficient of growth control by that substrate.

For important growth substrates, the substrate concentrations at which this control coefficient significantly exceeds zero, are so low that it is experimentally unfeasible to maintain them constant throughout an experimental definition of the growth rate, especially because the latter requires the cells to reach a steady state. Moreover, a better way to grow cells under steady-state conditions is to grow them in a chemostat (Monod 1942). In a chemostat, however, it is the growth rate, rather than the substrate concentration, that is set by the experimenter; parameter and variable are reversed.

In chemostats then, the pertinent experiment is to manipulate the growth rate (by manipulating the dilution rate) and measure the relative change in concentration of the growth limiting substrate. Because of the small value of this concentration, this is usually quite difficult, but for a limited number of cases this has now been accomplished (see Van Dam & Jansen 1991 for review). This then will give a coefficient for the control of substrate concentration in the chemostat (denoted as [X]) by the growth rate: $C_D^{[X]}$. The inverse of this coefficient is numerically equal to the coefficient of control of growth rate by the substrate concentration, as measured in the non-chemostat experiment. The proof of the latter property is as follows. Let us consider a small increase in dilution rate of the chemostat. $C_D^{[X]}$ now is the relative change in concentration of the growth limiting substrate in the culture, divided by the relative change in dilution rate: dln[substrate]/dlnD. Looking at it from the perspective of the cells, the substrate concentration has increased and they have responded by proceeding to a new growth rate. For the cells the situation must, in principle, be quite the same as that in a batch culture where the substrate concentration, kept at a fixed magnitude, is now kept at a slightly increased value. They adjust their growth rate. Therefore, $C_S^J$ is equal to dln|J|/dln[substrate]. Because D and J are equal, $C_S^J = 1/C_D^{[X]}$.

An alternative way of looking at the control of growth rate in a chemostat is to add the influx pump as a first reaction ('reaction 0') to the metabolic pathway (see Fig. 1B) with the property of having zero elasticity coefficients with respect to all metabolite concentrations. This first reaction then sets the constant flux (i.e., growth rate), and makes the pathway substrate an internal variable. Metabolic control analysis, including the calculation of control coefficients from elasticity coefficients, may now be performed. First one writes the matrix E:

$$\mathbf{E} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 1 & -\varepsilon_X^1 & -\varepsilon_Y^1 & -\varepsilon_Z^1 \\ 1 & -\varepsilon_X^2 & -\varepsilon_Y^2 & -\varepsilon_Z^2 \\ 1 & -\varepsilon_X^3 & -\varepsilon_Y^3 & -\varepsilon_Z^3 \end{bmatrix}$$

Inversion of this matrix gives the matrix that gives all control coefficients:

$$\mathbf{C} = \begin{bmatrix} C_D^J & C_1^J & C_2^J & C_3^J \\ C_D^X & C_1^X & C_2^X & C_3^X \\ C_D^Y & C_1^Y & C_2^Y & C_3^Y \\ C_D^Z & C_1^Z & C_2^Z & C_3^Z \end{bmatrix}$$

$C_D^J$ quantifies the control exerted by the pump (set at dilution rate D) on the steady-state growth rate J. Because of the zeros in the matrix **E**, the control matrix bears zeros at the same positions. This implies that $C_D^J = 1$ and $C_i^J = 0$ for any i; all control on growth rate lies in the pump and no control resides in any of the enzymes in the bacteria. Indeed, if in a chemostat the activity of an 'important' enzyme in the bacteria was increased (e.g., by addition of IPTG in the case of an operon under the control of the *lac* promoter), the growth rate of the bacteria in the newly attained steady state would not change; it would still equal the dilution rate of the chemostat. What would change of course is the concentration

of the growth substrate in the chemostat; $C_i^X$ does *not* equal zero; in a chemostat the cellular enzymes do control the concentration of the growth substrate, as they continue to control the concentrations of metabolites. It should be noted that in the definition of control coefficients used here, the change in enzyme activity is supposed to be the same for all individual bacterial cells. If, in contrast, one of the cells were to mutate, then its growth rate would be enhanced and it could still out-compete the wild-type cells, although again, its ultimate steady-state growth rate (after finishing the competition) would return to the preset dilution rate of the chemostat. In a chemostat, all control on growth rate resides in the pump and cellular enzymes do not control the growth rate in a chemostat. Under comparable conditions in batch cultures the same enzymes would control growth rate. In either case, we note that the dynamics of these systems are sufficiently complex that it is doubtful that a 'true' steady state is attained (Kell et al. 1991).

The above is an illustration of a potentially powerful aspect of MCA: because its definitions are so akin to definitions used in technological process optimization, its analyses can be made congruent with analyses of the process optimization of bioreactors. Indeed, the technological processes around the bioprocess (in this simplest example represented by the dilution rate D) can be taken into account by describing them as additional 'metabolic' processes. For instance, one may define, measure, and come to understand a coefficient of control of the aeration apparatus on the intracellular ATP concentration or on the concentration of the relevant bioproduct.

In the biotechnological context it is relevant to ask if MCA may be used in studies meant to increase yields or efficiencies of biotechnological processes. Previously we have indicated a strategy for such optimizations (Kell & Westerhoff 1986 a, b; Westerhoff & Kell 1987). If the interest lies in obtaining an intermediary metabolite of a microbe, then one should first obtain an estimated map of the metabolic pathway of interest. Subsequently, one should obtain estimates of kinetic properties of the enzymes in the pathway of interest. Rather than extensive knowledge of all kinetic properties, it suffices to know the so-called elasticity coefficients of the enzymes with respect to the metabolites. These elasticities correspond to the kinetic orders by which the rates depend on the metabolite concentrations, i.e., for a normal, far-from equilibrium Michaelis-Menten enzyme the elasticity for the substrate is $K_m/([S]+K_m)$, i.e., 1 far below the $K_m$ and approaching zero far above the $K_m$. One then puts this information into a matrix of elasticity coefficients (see above for an example), inverts this matrix, and obtains the coefficients for the control of pathway flux and metabolite concentrations by pathway enzymes. Above we demonstrated this procedure for the pathway of Fig. 1A. The chemostat may be analyzed by using the extended pathway of Fig. 1B, as also illustrated above. The enzymes that have the highest control coefficients with respect to the metabolite concentration of interest (or with respect to production rates of metabolites, though, in a chemostat, not with respect to growth rate, see above) are the candidates for genetic or other engineering, either by modifying their intracellular concentration or their elasticity coefficients (Kell & Westerhoff 1986 a).

Engineering approaches that affect more than a single enzyme at the same time can be dealt with similarly (Westerhoff & Kell 1987). The MCA as illustrated above for Fig. 1 yielded a matrix **C** of control coefficients as the inverse of a matrix **E**. For a manipulation that affects the activity of more than a single enzyme, one may write the change in enzyme activities as a column vector $d\ln(e_1)$, $d\ln(e_2)$, $d\ln(e_3)$. The change in variables that occurs when that change in enzyme activities is implemented is then given by the matrix product of **C** and this column vector:

$$\begin{bmatrix} d\ln|J| \\ d\ln X \\ d\ln Y \end{bmatrix} = \mathbf{C} \begin{bmatrix} d\ln e_1 \\ d\ln e_2 \\ d\ln e_3 \end{bmatrix}$$

This property may also be used in the reverse sense. If one wishes to change the microbes' metabolism, say the pathway flux by 10%, the concentration of X by −5%, leaving the concentration of Y unaffected, one may substitute these values for the

vector on the left hand side of the above equation and then invert the equation to read:

$$\begin{bmatrix} \delta \ln e_1 \\ \delta \ln e_2 \\ \delta \ln e_3 \end{bmatrix} = \mathbf{E} \begin{bmatrix} 0.10 \\ -0.05 \\ 0.00 \end{bmatrix}$$

where we used the fact that **E** equals the inverse of the **C** matrix. The left hand side of the above equation gives the best first-order estimates of the changes in enzyme activities one should establish in order to obtain the desired change in the microbe's physiology. The above example demonstrates how one can in principle calculate how to manipulate the cell in order to produce a desired metabolic phenotype. Although, obviously, the calculation has its limitations, it seems too simple *not* to make it before one embarks on extensive projects of genetic engineering.

## 4. Limits to conventional metabolic control analysis

*Enzyme activities regulated by other pathways are not considered*

As described above, metabolic control analysis was developed for the purpose of the analysis of the control of pathways of intermediary metabolism. Such pathways were conceptualized as a set of enzymes present at fixed activities (concentrations), acting on metabolites whose concentrations were freely variable. The steady-state metabolite concentrations and the pathway flux(es) are then a function of the enzyme concentrations, and it is the latter functional dependence that is quantified by the control coefficients.

In microbial physiology, the conceptual framework in which the enzyme concentrations are constant is valid only for short-term phenomena. For time-windows exceeding say 5 minutes, enzyme concentrations are likely to change due to changes in their rates of transcription, translation or degradation, and such changes are often relevant for the regulation of metabolic pathways. Well-known examples of the latter are the induction of the *lac* operon by lactose and the repression of the *his* operon by histidine. The corresponding type of regulation has rarely been considered in MCA (but see Barthelmess et al. 1974; Westerhoff et al. 1990), though it has been considered in Biochemical Systems Analysis (e.g., Savageau 1976).

Regulation of the *amount* of enzyme is not the only phenomenon that interferes with the conventional concept of MCA. It is also assumed that activation or inactivation of an enzyme, e.g., through covalent modification in reactions catalyzed by other enzymes, do not occur as part of the internal variation of the system. However, very notable examples of this are found in the signal transduction pathways of both eukaryotes and prokaryotes.

The recognition that regulated gene expression and covalent enzyme modification is not yet part and parcel of MCA raises a number of questions:

- Is it possible to extend MCA so as to include these phenomena?
- Are the laws that govern metabolic regulation also applicable if regulation through variable gene expression or covalent enzyme modification occurs in parallel?
- Is it possible quantitatively to weigh the relative importance of regulation at the metabolic, gene-expression and covalent enzyme modification levels?

We believe that the answers to these questions are in the affirmative, and will shortly (section 5) seek to show this with respect to the glutamine synthetase regulatory cascade of *Escherichia coli*. There is an additional problem with MCA, however, and that is that in principle it considers only small changes in system parameters.

*Large changes are not considered*

As reviewed above, MCA discusses control of physiology in terms of control and elasticity coefficients. These are defined in mathematical terms as derivatives of effects with respect to causes. Such derivatives may be translated into magnitudes of effects divided by the magnitude of their causes for

very (in fact infinitesimally) small effects. The advantage of definitions in terms of derivatives are that they are (i) unambiguous and (ii) they facilitate application of mathematics, such that control laws can be deduced that specify the connections between the control coefficients.

However, in many actual cases of regulation, changes are not truly small; they may well amount to 200% rather than to 1%. In such cases, MCA is only a first-order approximation of the actual status of the control of the pathway (still better than the conventional qualitative analyses). Second order extensions to MCA have been developed, but are fraught with complexity. The question therefore is: are there alternative methods to MCA that deal with substantial changes during regulatory transitions in a better-than-first-order approximation? Below we shall discuss two of these approaches, called Mosaic Non Equilibrium Thermodynamics and Biochemical Systems Analysis. First however, we shall deal with the possibility that enzyme activities and/or concentrations vary as a function of changes other than those made directly by the experimenter.

## 5. Modular metabolic control analysis

*The control of the regulatory cascade of glutamine synthetase in E. coli as a model system*

As we discussed in section 4, standard MCA discusses a physiological system as a single network in which all reactions are connected. In actual practice, cellular physiology is more organized than that. Several levels can be distinguished: the level of intermediary metabolism, the level of protein metabolism (synthesis, modification and degradation), the level of mRNA metabolism (transcription and decay). At the level of protein metabolism one can observe cascades of enzymes covalently modifying one another.

Conceptually, biochemists and cell physiologists have tended to separate the various levels of regulation of metabolism. Control is said to be at the level of transcription, at the level of translation, or 'just' metabolic. Conventional MCA did not acknowledge such a separation.

Recently MCA has been developed so as to analyze the contribution of the various controlling levels. Most explicitly this was done for systems with variable gene expression and for regulatory cascades: the modular metabolic control theory (Westerhoff et al. 1990; Kahn & Westerhoff 1991). The latter approach started from the general formalism of Reder (1988) and has the advantage of providing a better understanding of the control of the hierarchical levels of a cascade. Here, the modular control theory will be made explicit for the glutamine synthetase regulatory cascade, which is a complex and interesting system controlling the assimilation of $NH_4^+$ in enteric bacteria (review: Rhee et al. 1988).

Glutamine synthetase (GS) catalyses the incorporation of ammonium into glutamate resulting in glutamine. The activity of GS can be modified by the enzyme adenylyl-transferase ($AT_a$) to produce the less active form GS-AMP. The same enzyme catalyses both adenylylation and deadenylylation ($AT_d$) of GS. The transferase activity is stimulated by the regulatory protein $P_{II}$, while the deadenylylase activity is enhanced by the uridylyl form of $P_{II}$ ($P_{II}$-UMP). The modification of $P_{II}$ is catalyzed by uridylyl-transferase ($UT_u$), which has also, in an analogy to adenylyl-transferase, a deuridylylation activity ($UT_d$). $UT_u$ and $UT_d$ are regulated allosterically by α-ketoglutarate and by glutamine. Thus UT is a sensor for the nitrogen status of the cell. This cascade can be divided into three modules (see the boxes in Fig. 2). Each module contains a set of reactions which are connected with each other, but not with reactions from other modules. Therefore separate modules interact solely via effector-type interactions, i.e. substances from one module may affect reactions in another module without being a substrate or a product in this other module. Thus, in Fig. 2, both α-ketoglutarate and glutamine (which are in module 3) act as positive allosteric effectors of, respectively, $UT_u$ and $UT_d$, whilst glutamine is a negative effector of $UT_u$, in module 1. The structure of this metabolic network may be summarized in terms of a stoichiometry matrix **N** (see e.g. Reder 1988; Holstein & Greenshaw 1991), which consists of the stoichiometric coefficients of the reactions of the network. To clarify the mean-

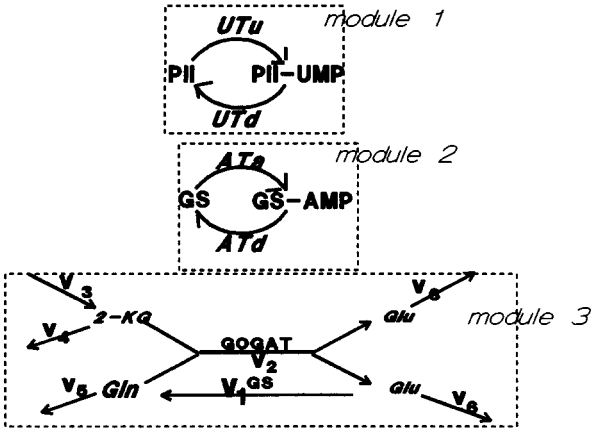


*Fig. 2.* Scheme of the pathways involved in ammonia assimilation in *E. coli*. 2-KG: α-ketoglutarate, Glu: glutamate, Gln: glutamine, GS: glutamine synthetase, PII: protein II, UTu: uridylyl transferase, UTd: deuridylylase, ATa: adenylyl transferase, ATd: deadenylylase, GOGAT: glutamine-2-ketoglutarate aminotransferase.

ing of this stoichiometry matrix for the glutamine synthetase regulatory cascade, let **x** be the column-vector of the molarities $[x_i]$ and **v** the column vector of the rates $[v_i]$; the time-dependent evolution of the system will be determined by the matrix product:

$$d\mathbf{x}/dt = \mathbf{N} \cdot \mathbf{v}$$

This can be expressed for the glutamine synthetase regulatory cascade (see Fig. 2). For the purpose of this article, we will simplify the treatment and consider that the GS/GOGAT cycle is the only route of ammonium assimilation, ignoring the glutamate dehydrogenase (GDH) route (as we would do with a glutamate dehydrogenase negative mutant). We obtain:

In the stoichiometry matrix, the modular structure of the metabolic network is indicated by the dashed lines. In shorthand, we may write:

$$\mathbf{N} = \begin{bmatrix} \mathbf{N}_1 & \mathbf{0} & \mathbf{0} \\ \mathbf{0} & \mathbf{N}_2 & \mathbf{0} \\ \mathbf{0} & \mathbf{0} & \mathbf{N}_3 \end{bmatrix}$$

where $\mathbf{N}_i$ indicates the stoichiometry matrix of the module of interest. Now we may build an elasticity matrix containing all elasticity coefficients (see section 2) of the system:

$$\boldsymbol{\varepsilon} = [\partial \ln|v_i|/\partial \ln x_j]$$

Here ∂ refers to partial differentiation. Each coefficient $\varepsilon_{ij}$ of the elasticity matrix $\boldsymbol{\varepsilon}$ quantifies the effect of molecular species *j* (at molarity $x_j$) upon rate $v_i$. The elasticity matrix $\boldsymbol{\varepsilon}$ of the glutamine synthetase regulatory cascade is decomposed into blocks according to the modular structure of the system, as mentioned above:

$$\boldsymbol{\varepsilon} = \begin{bmatrix} \boldsymbol{\varepsilon}_1^1 & \mathbf{0} & \boldsymbol{\varepsilon}_3^1 \\ \boldsymbol{\varepsilon}_1^2 & \boldsymbol{\varepsilon}_2^2 & \mathbf{0} \\ \mathbf{0} & \boldsymbol{\varepsilon}_2^3 & \boldsymbol{\varepsilon}_3^3 \end{bmatrix}$$

where $\boldsymbol{\varepsilon}_i^j$ (which is itself a matrix, hence written bold face) refers to module *1* (above), and so on. The block $\boldsymbol{\varepsilon}_2^1$ is null because the state of the GS interconversion cycle (module *2*) does not directly influence the rates of the $P_{II}$ interconversion cycle (module *1*). Similarly the block $\boldsymbol{\varepsilon}_1^3$ is null because $P_{II}$ and $P_{II}$-UMP do not directly influence the metabo-

$$\begin{bmatrix} dP_{II}/dt \\ dP_{II}\text{-UMP}/dt \\ dGS/dt \\ dGS\text{-AMP}/dt \\ d\alpha\text{-KG}/dt \\ dGlu/dt \\ dGln/dt \end{bmatrix} = \left[\begin{array}{cc|cc|cccccc} -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ \hline 0 & 0 & -1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 \\ \hline 0 & 0 & 0 & 0 & 0 & -1 & 1 & -1 & 0 & 0 \\ 0 & 0 & 0 & 0 & -1 & 2 & 0 & 0 & 0 & -1 \\ 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 & -1 & 0 \end{array}\right] \cdot \begin{bmatrix} v_{UTu} \\ v_{UTd} \\ v_{ATa} \\ v_{ATd} \\ v_1 \\ v_2 \\ v_3 \\ v_4 \\ v_5 \\ v_6 \end{bmatrix}$$

lic rates of module 3. In principle, the block $\boldsymbol{\varepsilon}_3^2$ differs from 0 because the GS interconversion cycle can also be directly influenced by the metabolic status. However, to simplify the presentation, this effect is neglected here. A more complete treatment will appear elsewhere (Kahn & Westerhoff 1991). The block $\boldsymbol{\varepsilon}_2^3$ is special, in that it contains the elasticities of the glutamine synthetase reaction towards GS and GS-AMP. If, for the sake of simplicity, we assume that GS-AMP is fully inactive, we can write:

$$\boldsymbol{\varepsilon}_2^3 = \begin{bmatrix} 1 & 0 \\ 0 & 0 \\ 0 & 0 \\ 0 & 0 \\ 0 & 0 \\ 0 & 0 \end{bmatrix}$$

This is because with the definitions for the rates given in Fig. 2:

$$\begin{bmatrix} d\ln|v_1| \\ d\ln|v_2| \\ d\ln|v_3| \\ d\ln|v_4| \\ d\ln|v_5| \\ d\ln|v_6| \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ 0 & 0 \\ 0 & 0 \\ 0 & 0 \\ 0 & 0 \\ 0 & 0 \end{bmatrix} \cdot \begin{bmatrix} d\ln[GS] \\ d\ln[GS\text{-}AMP] \end{bmatrix}$$

Similarly we can construct a matrix of control coefficients **C** containing all the flux control coefficients of the system, and decompose it into blocks following the modular structure of the system:

$$\mathbf{C} = \begin{bmatrix} \mathbf{C}_1^1 & \mathbf{C}_2^1 & \mathbf{C}_3^1 \\ \mathbf{C}_1^2 & \mathbf{C}_2^2 & \mathbf{C}_3^2 \\ \mathbf{C}_1^3 & \mathbf{C}_2^3 & \mathbf{C}_3^3 \end{bmatrix}$$

where the submatrix $\mathbf{C}_j^i$ contains the flux control coefficients describing the sensitivities of the fluxes within module *i* to changes in the process-activities in module *j*.

Now suppose we have studied the metabolic part of this system (module *3*) without operation of the regulatory cascade (that is, at a constant, clamped, level of GS adenylylation, equal to the steady-state level of GS adenylylation normally attained). We can analyze the control of such a subsystem and place the resulting control coefficients into an *intrinsic* control matrix which we will note $\mathbf{C}_3$. $\mathbf{C}_3$ will in general be different from the matrix $\mathbf{C}_3^3$ containing the control coefficients of module *3* when the regulatory cascade is left to operate (that is, the level of GS adenylylation is allowed to vary freely). Note, however, that the elasticity matrix of the metabolic module *3* (or of any other module) is an intrinsic property and therefore is $\boldsymbol{\varepsilon}_3^3$ both in the clamped and in the non-clamped system. Recently, we have been able to demonstrate a relationship allowing to calculate the matrix $\mathbf{C}_3^3$ from the knowledge of the intrinsic *flux* control matrix $\mathbf{C}_3$, the intrinsic *concentration* control matrices $\mathbf{S}_1$, $\mathbf{S}_2$ and $\mathbf{S}_3$, and the elasticity matrices $\boldsymbol{\varepsilon}_j^i$ (Kahn & Westerhoff 1991, **I** is the identity matrix):

$$\mathbf{C}_3^3 = \mathbf{C}_3 \cdot (\mathbf{I} - \boldsymbol{\varepsilon}_2^3.\mathbf{S}_2.\boldsymbol{\varepsilon}_1^2.\mathbf{S}_1.\boldsymbol{\varepsilon}_3^1.\mathbf{S}_3)^{-1}$$

Here it has been assumed that module 3 does not directly affect module 2. The first term of this product is the *intrinsic* control matrix of the metabolic module, whereas the second term describes the effect of the cyclic regulation on the control within the module. Thus the control matrix can be calculated as the product of an intrinsic control matrix and a regulatory term referring to the regulation through the other modules. Similarly, this can be done for the concentration control coefficients. The importance of these relationships is at least twofold: (i) the regulatory effects of a process on a flux in the same module are equal to the regulatory effects if the module were in isolation, divided by a term that measures the regulatory effects through all other modules (and that goes to 1 if those other effects are absent or incompletely connected), and (ii) it is possible to determine the control properties of the modules separately before constructing the control of the entire system. In this manner, modular metabolic control analysis marries reductionism and holism (see section 7).

Moreover, the above modular control analysis allows one to quantify the *regulatory strengths* (Kahn & Westerhoff 1992) of the cascade, which express quantitatively the sensitivities of nitrogen assimilation fluxes to fluctuations in the levels of $P_{II}$

uridylylation. These are the elements of the following *regulation matrix*:

$$\mathbf{R}_1^3 = \mathbf{C}_2^3.\boldsymbol{\varepsilon}_1^2 = \mathbf{C}_3^3.\boldsymbol{\varepsilon}_2^3.\mathbf{S}_2.\boldsymbol{\varepsilon}_1^2$$

It is the product of an elasticity matrix $\boldsymbol{\varepsilon}_1^2$ expressing the response of the GS adenylylation cycle to changes in $P_{II}$ uridylylation, and of a control matrix $\mathbf{C}_2^3$ expressing the control of nitrogen assimilation fluxes by GS adenylylation (Kahn & Westerhoff 1991).

This brief account of how Modular MCA can be applied to the GS regulatory cascade indicates that the way is now open for the quantitative analysis of rather more complex systems than was heretofore possible. First, one decomposes the system into its relevant disconnected modules. Second, the control of each module is analyzed individually, by clamping the concentrations in the other modules. Third, the complex system is analyzed as a whole, both experimentally and by mathematical reconstruction. If experiment and mathematical reconstruction are consistent, one may consider that the model on which the latter was based is a sound quantitative model. Thus Modular MCA is a method for treating the control of complex metabolic systems by exploiting their structure, when the completely direct treatment is too complicated.

## 6. Larger changes: biochemical systems theory and mosaic non equilibrium thermodynamics

The elasticity and control coefficients of MCA are defined as derivatives, i.e., as the ratios of infinitely small changes. In practical applications, these are replaced by ratios between small changes. However, as discussed in section 4, for many cases of interest in microbial physiology, larger changes are important. Why not then use the same definitions and theory for larger changes? Thus one might be inclined to define the coefficient of the control of enzyme *i* exerted on flux J as the percentage decrease in flux divided by the percentage reduction in activity of that enzyme. However, the magnitude of such a control coefficient would almost always depend on the percentage change in enzyme activity. And, for a linear pathway, the flux control coefficient of each enzyme would tend to approach 1 when the inhibition of activity approached 100%, since complete elimination of that enzyme will reduce the pathway flux to zero. Indeed, the magnitude of the sum of all the control coefficients would depend on the magnitude of the percentage change in enzyme activity made in the determination of the control coefficients.

Up to this moment, no complete solution for this dilemma has been found. What is left for the analysis of larger changes is:

(i) just use MCA and accept the result as approximate for the description of the actual control and regulation
(ii) perform a complete integration of all the kinetic equations of the system
(iii) use approximating, but simpler descriptions
(iv) use methods of artificial intelligence (Kell & Davey 1991).

The disadvantage of approach (ii) is that it requires the precise knowledge of most kinetic characteristics of the system. In addition, because of the incongruence between standard integration subroutines and the structure of metabolism and physiology, this procedure tends to lose touch with biochemistry. Object-oriented programming approaches have recently been used in attempt to alleviate the latter problem (Stoffers et al. 1991).

The third method has been applied, at times with considerable success. This degree of success may seem somewhat surprising as it would seem impossible to approximate the richness of the kinetics of cellular reactions by simpler rate equations and still simulate cellular behavior. However, the success becomes more understandable if it is granted that we are generally less interested in understanding the *precise* quantitative behavior of physiological systems than in understanding the *essence* of such behavior, even though the latter may include quantitative aspects (such as synergism) (Savageau 1976).

Non Equilibrium Thermodynamics (NET) has been an approach, which, although its accuracy in

describing all the kinetic features of biochemical kinetics is severely limited, could still make one understand the essence of phenomena such as free-energy transduction, coupling, and optimal states (Caplan & Essig 1983; Stucki 1980; Westerhoff & Van Dam 1987). For microbial growth this led to insights into why the efficiency of microbial growth may be as low as it is (Westerhoff et al. 1983; see however Heijnen 1991). A variant of NET, called Mosaic Non Equilibrium Thermodynamics (MNET) was developed so as to remove the most apparent inconsistencies between NET and biochemical kinetics (reviewed: Westerhoff & Van Dam 1987). It has been applied to enhance understanding of what underlies the phenomenon of growth rate-dependent and growth rate-independent maintenance metabolism (Hellingwerf et al. 1982), as well as the basis for the distinction between Carbon- and Energy-limited growth (Westerhoff & Van Dam 1987). The most recent review of this method may be found in Rutgers et al. (1991).

MNET may be considered an extension of a branch of MCA that focuses on the control aspects of free-energy metabolism (Westerhoff & Van Dam 1987). Biochemical Systems Analysis (BST) is a method that is parallel to MCA. Its basic approach may be rationalized as follows: If one describes the dependence of a reaction rate on its substrate concentration by an elasticity coefficient, and one assumes that the elasticity coefficient does not change much as the substrate concentration is increased, then one may integrate and describe a reaction rate by:

$$v = k \cdot [S]^{\varepsilon_S}$$

Transition to logarithmic space allows one to integrate systems with this type of rate equations in a simple way (Savageau 1976; Voit 1991). A problem arises when groups of reactions are aggregated or when reversible reactions are considered, but even for those cases the approximation has been shown to work reasonably well (Voit & Savageau 1987; Voit 1991). Up to this moment BST has been used to describe the general behaviour of biological systems and indeed, qualitative conclusions of general value have been attained. The application of the method in direct experimentation has remained limited, because the basic definitions in BST are somewhat remote from experimental observables, in contrast to the definitions of MCA and MNET (see however, Groen & Westerhoff 1990).

## 7. Concluding remarks

*Methods to reduce complexity: the rationalization of reductionism*

In this paper we have discussed a number of modern approaches to the quantitative analysis of microbial physiology. This should be regarded as a parallel to the review of concrete applications of these methods by Van Dam & Jansen (1991).

It is often suggested that the sole aim of quantitative methods should be to describe, accurately to the second decimal place, rates and concentrations in systems. We take issue with this. The more important aim of these methods is to realize a basic tenet of biochemistry and biophysics, i.e., that, in principle biology should be explicable in terms of physical and chemical principles. And, 'explicable' should mean 'explicable in the physical chemical sense', i.e., in principle including the quantitative detail.

Too often, the latter tenet has been subject to immaterial debate between holists and reductionists, the latter emphasizing studies of single mechanisms that occur in cells, the former stressing that doing so destroys the essence of cellular, organismal and ecological organization. The methods we have discussed here provide a scientific link between molecular mechanisms (elasticity coefficients) and properties of the cell as a whole (control coefficients). Indeed, laws such as the summation and the connectivity theorems (see above) are the very expression of the fact that the whole is more than the simple sum of its parts.

Of course, we note that, to date, most of these types of analyses take into account only limited aspects of cellular organization. Organization on the basis of metabolic channelling (e.g. Welch & Keleti 1990) is not usually considered (see however Kell & Westerhoff 1985, 1990; Westerhoff and Kell

1988; Kacser et al. 1990; Ovádi 1991), and neither are phenomena, often called 'self-organization', which invoke bistability and hysteresis (Nicolis & Prigogine 1977; see however Cortassa et al. 1991). In truth, it is not always easy, and may not be possible in principle, to put Humpty Dumpty back together again (Kell & Welch 1991).

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