



## What BioTechnologists Knew . . . ?

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*“Nothing is more difficult to undertake, more perilous to conduct, or more uncertain in its outcome than to take the lead in introducing a new order of things, for the innovator has for its enemies all those who have done well under the old, and lukewarm defenders who may do well under the new”*

Machiavelli, “The Prince”

Qualitative, trial-and-error methods designed to increase the flux to desirable biotechnological products have led to new technologies and vast improvements in existing ones. However, these methods now appear in many cases to have approached their limit. In addition, there is a strong feeling in industry that much of the recent boom in academic knowledge of biochemistry and molecular biology passes biotechnology by, simply because one cannot evaluate the implications of molecular kinetics for the functioning of the producer organisms as a whole. New methods, or more rational methods, are called for. One, aimed at increasing only the concentration of a single metabolite by site-directed mutagenesis is developed here.

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### Introduction

We here review what has become of the insights of Henrik Kacser into how Metabolic Control Analysis (MCA) provides the necessary rational approach to bioengineering. The control coefficients point at the enzymes that need to be amplified to increase a desired flux, yield or concentration. Kinetic properties, called elasticity coefficients, can be used to calculate the control coefficients, and a variety of experimental methods have been developed for the direct measurements of the latter. MCA can further be used to calculate the combination of changes in enzyme activities that should be engineered in order to fulfil a requirement of a number of simultaneous changes. MCA is no longer limited to “ideal”, “academic” metabolic pathways: signal transduction, regulated gene expression, metabolite channelling and cellular dynamics are all within reach. In the “universal method” as developed by Kacser and

co-workers, this approach is not necessarily limited to very small changes. We here elaborate this method somewhat.

Where the previous MCA-based engineering principles focused on the modulation of enzyme concentrations, we here describe a strategy using site-directed mutagenesis. This strategy aims at increasing specifically the concentration of certain metabolites or certain fluxes without causing changes in any other metabolite or flux, hence safeguarding cellular homeostasis. The approach could also be useful when large changes are desired.

The principles of MCA are ready for industry, but how ready is industry for this rational approach to bioengineering?

### The Aims of Bioengineering

Bread, beer, wine, cheese and yoghurt are well-known examples of how important biotechnology is even for modern man. The optimisation of the production processes of these foods is still a subject

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of considerable interest if only in terms of taste and stability. Developments in microbiology, biochemistry and genetics have opened up a gamut of other (potential) applications of biotechnology: Biochemistry demonstrated that living cells produce an enormous variety of chemical substances and that it was possible to identify these substances, their biosynthetic routes and the catalysts that are essential to the latter. Microbiology showed that on the one hand there is an enormous variety of microorganisms with different metabolic patterns and therefore with different potential for product formation (Kell *et al.*, 1995), whereas on the other hand all these organisms are rather similar in terms of their central biochemistry and biophysics. Classical genetics showed that one may count on the forced or natural occurrence of mutations and then select strains with improved properties. Molecular genetics, now including such combinatorial approaches as DNA shuffling (Stemmer, 1994a,b) and sequencing by hybridisation against huge oligonucleotide arrays (Lipshutz *et al.*, 1995), made it and will make it possible to improve the genetic properties in a directed fashion and to cross barriers between species. In fact then, the producer microorganism seemed to have become a micro-workshop, in which all tools and production lines can be made available for the production of virtually anything desirable.

To a considerable extent the strategy of bioengineering we have just described has been successful. Yet, limitations to the approach have surfaced. The micro-workshop appeared more like a factory of considerable complexity, which required special management, and which in fact already possessed management directed at purposes quite different from what the biotechnologist desires. Metabolic pathways turn out to be regulated (unsurprisingly) for the optimum survival of the cell, and such regulation is likely to differ from that leading to maximal productivity. When the biotechnologist amplifies a gene encoding an enzyme that is required for a metabolic flux, that enzyme may turn out to be crucial yet not significantly controlling the flux; homeostatic responses of the cell may compensate the amplification, or the control may readily shift to some other factor.

There seem to be at least two ways to deal with those limitations to bioengineering. One is to replace the cell's own management by a new management structure, e.g., by inactivating regulatory proteins and substituting others for them. Another is to make use of the cell's own management in a clever way and trick the cell into producing more of the product of interest without being disturbed sufficiently to begin to invoke homeostatic responses. Either approach is

a fascinating and complicated challenge, and involves detailed and quantitative understanding of how the cell controls its own function. Yet neither approach involves a "free lunch", and there is no room for rule-of-thumb estimates, except as starters of subsequent, quantitative elaborations. Cell management is an example of BioComplexity and involves the simultaneous operation of numerous regulatory effects with different quantitative strengths and of different directions. Only a quantitative analysis can properly evaluate the resultant of the intermediary effects (Kell & Sonnleitner, 1995). Such an analysis is what we call a rational approach to bioengineering (Kell & Westerhoff, 1986a,b) and, especially when enzyme levels are manipulated by cloning (Skatrud *et al.*, 1989), is nowadays widely promoted as "metabolic engineering" (e.g. Bailey *et al.*, 1990; Bailey, 1991; Stephanopoulos & Vallino, 1991; Cameron & Tong, 1993; Katsumata & Ikeda, 1993; Stephanopoulos & Sinskey, 1993; Mermelstein *et al.*, 1994; Simpson *et al.*, 1995).

### The Role of MCA

How can one bring about the quantitative understanding of cell management necessary for rational bioengineering? Garfinkel and colleagues began with an invaluable integral model of significant parts of cell metabolism (Kohn *et al.*, 1979). However, their approach was too ambitious for its time. Essentially these authors aimed at being able to calculate the behaviour of their metabolic network in full quantitative detail, over the entire range of all possible states. Even at present such an aim is overly ambitious, although some do not appear to recognise this (Maddox, 1994). The trouble was that Garfinkel and colleagues included every known detail of every component of the system into their model, which thereby became too complex and too undetermined (because too few of the kinetic details were known with sufficient precision). Their problem was that they were also fitting multivariate data to a very complex system of many parameters, a problem which frequently has many apparently equally good solutions, even in the absence of serious experimental noise. Hence there is a problem as to what is in fact the "right" solution. Other authors had recognised these problems from the onset, and started with simplifications, such as assuming linear rate equations for all reactions (e.g., implying that all substrate concentrations are far below the corresponding Michaelis constants) (Heinrich *et al.*, 1977), or by assuming Onsager reciprocal and proportional, non-equilibrium thermodynamic flow-force relationships (Katchalsky & Curran, 1967). The models of

these authors met with great scepticism from the biochemists, since the biochemists were precisely interested in those nonlinear properties of the enzymes which they considered most likely to give rise to the interesting regulatory phenomena then being elucidated. Attempts to bridge the gap between these modellers and the biochemists included the approaches of Mosaic Non Equilibrium Thermodynamics (Westerhoff & Van Dam, 1987) and Biochemical Systems Theory (Savageau, 1976). These two approaches implemented approximations of the true rate equations, which kept some of the nonlinear characteristics of the individual rate equations and allowed mathematical integration of all individual rate equations into systemic rate equations. These approaches have also been met with scepticism, perhaps because too much of the biochemistry was felt to be approximated away (see however, Senn *et al.*, 1994), or just because the approaches involved more mathematics than most biochemists were comfortable with.

It was the accomplishment of Higgins (1965), Heinrich & Rapoport (1973), Savageau (1976), but most of all of Kacser & Burns (1973), that a fundamentally different approach was also taken. Rather than continuing to ask the most ambitious question of quantitatively understanding the integral behaviour of the system but using approximations in the approach, Kacser & Burns retreated to a more modest type of question, which could be answered exactly. They were satisfied by "only" understanding quantitatively how a steady state changes, when one of the parameters is perturbed only slightly, provided that the understanding be precise. The object of understanding was given a quantitative form in terms of the Control Coefficients. Kacser & Burns achieved this understanding for the first time when they derived the summation and connectivity theorems for flux control and implemented these for a linear pathway. In this manner they could express the flux-control coefficient of any of the enzymes in terms of the elasticity coefficients of all the enzymes. Epistemologically, this has been a crucial point in the development of the field, not only because the modest ambition of understanding flux control in terms of enzyme properties had been accomplished, but also because it showed that not all kinetic and thermodynamic properties of the enzymes were needed, but only one or two particular or compound *aspects* of those properties, known as the elasticities (Burns *et al.*, 1985) with respect to substrates, products and modifiers.

As he did with so many others, Henrik inspired us greatly. His own drive was to understand why in classical genetics so many genotypes were phenotypi-

cally silent. His explanation was that a heterozygous organism containing only 50% of the active form of a gene, and hence probably only 50% of the corresponding enzyme, should be inhibited by much less than 50% in its functions, because the control by each enzyme should not equal one but the sum of the control by *all* enzymes should. For ten enzymes in the pathway, this would result in an average control of 10% and an average effect of heterozygous deletions of 5%, essentially unnoticeable experimentally (Kacser & Burns, 1981).

In the early 1980s we became interested in applying the above described challenge to biotechnology [and in using it to improve our understanding of the properties of proton-coupled bioenergetic systems (Kell & Westerhoff, 1985; Westerhoff *et al.*, 1985)]. Henrik's inspiration made us realise that biotechnology should go for control coefficients, i.e., for the extent to which one can increase the flux or metabolite concentration of interest by activating any of the enzymes (Kell & Westerhoff, 1985, 1986a,b; Westerhoff & Kell, 1987; Kell *et al.*, 1989; Rutgers *et al.*, 1991; Westerhoff *et al.*, 1991; Van Dam *et al.*, 1993). Accordingly, it should be highly relevant for biotechnologists wishing to increase the flux to a desirable metabolite of interest to know how to estimate the magnitude of the control coefficients from the kinetic properties of the enzymes in the system (or indeed by any empirical approach).

The response of many in the biotechnology field was as has often been quoted in other contexts by Henrik Kacser. At first it was considered not true that the amount of control exerted by an enzyme was determined by the elasticity coefficients. For, had it not already been known for a long time that the first irreversible step in any metabolic pathway was the rate-limiting step? And if one wished to overcome this, that it sufficed to amplify that enzyme? Worrying about the control exerted by any other enzyme in the pathway, let alone all enzymes, was considered an academic waste of time (notwithstanding that it was recognised that most amino acid overproducers were *regulatory* mutants resistant to feedback).

Subsequently, after much preaching of the gospel by the "pope" himself and by some of the cardinals (as Henrik would say), and after experimental demonstrations such as that by Groen and colleagues (1982) which showed unequivocally that the control of mitochondrial respiration was distributed, some biotechnologists admitted that flux control can be distributed, but considered it unimportant. They *were* improving the organisms by random mutagenesis anyway (or occasionally by directed mutagenesis) and that did work, did it not?

Amplifying phosphofructokinase in yeast does not affect glycolytic flux (as discussed in Westerhoff, 1995), and hence the implications of Metabolic Control Analysis (MCA) are important: amplification of an essential and regulated enzyme in a pathway need not much increase the flux. This however, is what biotechnologists knew all along†, or is it?

Indeed there is something to be said for the position that “we knew it all along”, since the notion that a system as complex as a regulated metabolic pathway does *not* have a single rate-limiting step, the same under all conditions, is almost intuitive. The concept that there should be a single rate-limiting step, far-from-equilibrium, is much more of a theoretical construct. However, these systems are too complex, and our criteria for scientific rigour too high, for us to rely on intuitive solutions. This perhaps is where approaches such as Metabolic Control Analysis have their greatest value: they enable us to approach metabolic control and regulation scientifically.

### Shortcomings of MCA and Their Resolution

In fact there is an additional phase of “recognition” of MCA by biotechnologists. After stating that they knew it all along, biotechnologists began to lay stress on the perceived (and in some cases true) limitations of MCA, such as that MCA be limited to linear pathways at steady state, that metabolite channelling and regulated gene expression invalidated MCA, that cellular systems were too complex anyway to be dealt with in terms of MCA, that MCA dealt with control but not with regulation, that MCA could only deal with small (infinitesimal) changes, whereas biotechnology required substantial improvements of productivity, that MCA dealt only with systems with fixed substrate concentrations, whereas in continuous culture the growth rate rather than the substrate concentrations is fixed, and that MCA tends to treat systems as homogeneous whereas the cell suspensions typically studied are highly heterogeneous, as may be determined by flow cytometry (Kell *et al.*, 1991; Davey & Kell, 1996). However, from its very beginning MCA had not been limited to linear pathways. Hofmeyr *et al.*, 1986 (cf. Westerhoff & Chen, 1984; Westerhoff & Van Dam, 1987; Small & Fell, 1990; Kholodenko *et al.*, 1994) clarified remaining difficulties involving moiety conservation. The analysis of large systems has been simplified by modular approaches (Westerhoff *et al.*, 1987; Schuster *et al.*, 1993; Hafner *et al.*, 1990; Kholodenko *et al.*, 1995b). As to regulated gene expression

(Barthelmess *et al.*, 1974; Westerhoff & Van Workum, 1990; Kahn & Westerhoff, 1991; Hlavacek & Savageau, 1995; Jensen *et al.*, 1995), signal transduction (Kahn & Westerhoff, 1991; Kholodenko & Westerhoff, 1995a), and metabolite channelling (Westerhoff & Kell, 1988; Kell & Westerhoff, 1990; Kholodenko & Westerhoff, 1993, 1995a,b; Mendes *et al.*, 1996; see also Kell & Westerhoff, 1985; Sauro & Kacser, 1990) these limitations have since been removed. Dynamic phenomena can also be addressed by MCA (Acerenza *et al.*, 1989; Westerhoff *et al.*, 1990; Sauro, 1990; Markus & Hess, 1990; Heinrich & Reder, 1991; Liao & Delgado, 1993). Regulation vs. control has been discussed quite elegantly by Hofmeyr and co-workers (Hofmeyr *et al.*, 1993; see also Sauro, 1990; Hofmeyr & Cornish-Bowden, 1991; Kahn & Westerhoff, 1993) and MCA has been developed for chemostats (Small, 1994; Snoep *et al.*, 1994). And, for the most recent MCA, see Westerhoff *et al.* (1996).

### Effecting Large Increases in Flux

The initial limitation of MCA to small changes has been addressed in two ways. First, a second-order control analysis was developed, giving some insight into the extent to which the control shifts to other enzymes when one enzyme is amplified (Höfer & Heinrich, 1993; see also Westerhoff & Kell, 1988). This approach is tedious, however, as the second-order equivalents of the summation and connectivity theorems become overly complex (and is still only an approximation which can be almost as bad as the first order approach in some highly nonlinear systems).

Then, Henrik Kacser and co-workers contributed two leaps forward. The first leap was the generalisation of the control coefficient to the deviation index, more suitable for the description of large changes. Importantly, the deviation index was again defined as the change in flux divided by the change in enzyme concentration, but in this definition, both changes were normalised by the flux and enzyme concentration, respectively, *after* the change. This deviation index is often well approximated by the control coefficient, much better than if the changes are normalised by the flux and enzyme activity before the changes (Small & Kacser, 1993; Kacser, 1995). Using this method, the effects of large increases in enzyme activity are predicted quite well by the flux-control coefficient.

The second leap forward was called the “Universal Method”. It aimed at making large changes in a pathway flux, without changing any of the metabolite concentrations. For instance, if in Fig. 1(b) one third

† Freely adapted from William James.

of the flux flows to  $P_1$  and two thirds flow to  $P_2$  and one wishes to double the flux to  $P_1$ , one may effect this by doubling the activities of enzymes 3 and 4, and increasing the activities of enzymes 1 and 2 by one third (Kacser & Acerenza, 1993; Small & Kacser, 1994). We recognise, however, as did its authors, that the “Universal Method” in its present form cannot strictly be applied to systems in which the concentration of the product  $P$  is variable to the extent that it retro-affects the pathway, nor where moiety-conserved cycles are present.

### Changing the Concentration of One’s Favourite Metabolite

Much of the interest in the biotechnological implementations of MCA has focused on metabolic fluxes. In some cases, however, the actual interest may lie in a change in the concentration of a metabolite, either because that metabolite is itself valuable, or because it serves as a signal for bringing a certain process to a desired activity. Examples for the latter case could be cAMP or intracellular lactose.

How could one bring about a desired change in the concentration of a single metabolite without perturbing cellular metabolism, i.e. whilst keeping all other metabolite concentrations and all fluxes constant?

The proof of the connectivity theorem, as developed by Kacser & Burns (1973), may serve to inspire a solution to this problem that is much more intuitive than the more general solution given further below, but nevertheless tight, and, therefore, superior. The argument is due to Kacser with his colleagues Acerenza and Small (Kacser & Acerenza, 1993; Small & Kacser, 1994). In their proof Jim Burns and Henrik Kacser considered a change  $\delta X$  in the concentration of a metabolite  $X$ . They evaluated the change in

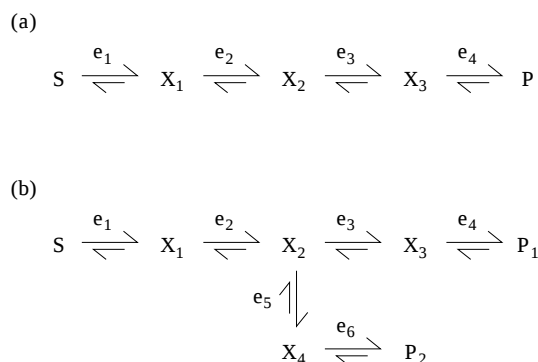


FIG. 1. Two simple metabolic pathways, linear (A) and branched (B). S, the P’s, and the enzymes ( $e_i$ ) are assumed to be present at constant concentrations, whereas the concentrations of the intermediary metabolites ( $X_i$ ) are freely variable.

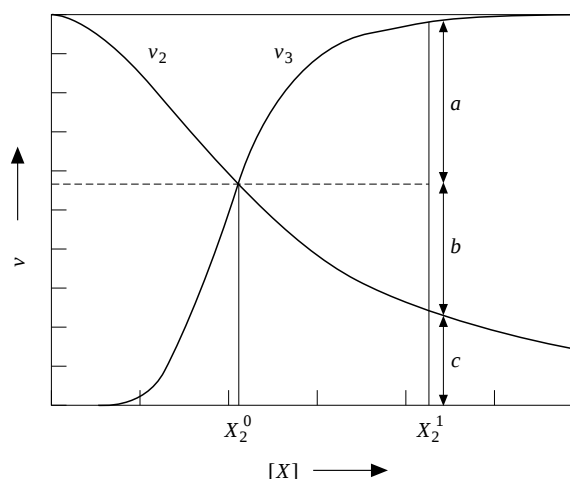


FIG. 2. Graphical method for calculating required changes in enzyme concentration for the accomplishment of large changes in the steady-state concentrations of a single metabolite  $X_2$ . See text for details.

rate of a reaction  $i$  by multiplying  $\delta X/X$  by the elasticity of reaction  $i$  with respect to  $X$ .

$$\delta \ln v_i = \varepsilon_x^i \cdot \delta \ln X$$

They then proposed to change the concentration of enzyme  $i$  by  $-\varepsilon_x^i \cdot \delta \ln X$ , such that the change in rate  $v_i$  was annihilated. Importantly, the authors realised that the system would again be at steady state, hence that no flux should have changed, hence:

$$0 = \delta \ln J = \sum_i C_i^J \cdot (-\varepsilon_x^i) \cdot \delta \ln X$$

which gives the flux-control connectivity theorem. The PhD thesis of Jim Burns contains the corresponding derivation of the concentration-control connectivity theorem (Kacser, personal communication). Neither Yi-der Chen nor HWV were aware of this when they (re)derived this theorem by way of this same proof plus two other proofs (Westerhoff & Chen, 1984).

For now, we shift attention to the fact that in the derivation of the connectivity theorem a new steady-state was attained in which only the concentration of  $X$  was changed. This is what we intend to achieve in this section of the present paper. Accordingly, if one wishes to engineer a microbial strain such that only the concentration of a metabolite  $X$  is changed by  $\delta X$ , one can accomplish this by changing the concentrations of all enzymes that are sensitive to changes in  $X$  by the fraction  $-\varepsilon_x^i \cdot \delta X/X$ . To increase  $X_2$  in Fig. 1(a) by 1%, one should activate enzyme 2 by  $\varepsilon_x^2\%$  and inactivate enzyme 3 by  $\varepsilon_x^3\%$ .

It is of great interest here that MCA is not necessarily limited to small changes. When a

substantial change in the concentration of a metabolite is desired, the differential analysis must be replaced by a difference analysis. In Fig. 2  $X_2^0$  is the concentration of  $X_2$  at the starting steady state and  $X_2^1$  is the desired steady state concentration. The desired concentration  $X_2^1$  will be obtained if the concentration of enzyme 2 is increased by the factor  $(b+c)/c$  and the concentration of enzyme 3 reduced by the factor  $(a+b+c)$ . Concentrations of other metabolites and fluxes will not be affected. Small & Kacser (1994) devised a non-graphical method based on the rate equations and mass action ratios.

One may note that we here deal with modulation of enzymes in terms of enzyme concentration. The more general formulation is in terms of enzyme activity (Schuster & Heinrich, 1992). For ideal systems (for definition of "ideal" see Kholodenko & Westerhoff, 1995b; Kholodenko *et al.*, 1995a) this gives identical results. For most non-ideal systems, the formulation in terms of activity gives the proper results, except for some more special cases (Kholodenko *et al.*, 1995b).

### Engineering Metabolism: the General Solution

Above we have focused on the homeostatic way of modulating an organism, i.e., on the aim of changing one flux or one metabolite concentration at constant magnitudes of all other concentrations and of all fluxes. These are in fact special cases of the question of how one can engineer a prescribed set of changes in all metabolite concentrations and all fluxes. For the case of small changes, the answer is simple in theory. Flux and concentration control coefficients have been grouped in a control matrix  $\mathbf{C}$ , such that for the pathway of Fig. 1(a) (Westerhoff & Kell, 1987; Sauro *et al.*, 1987; Westerhoff *et al.*, 1994):

$$\mathbf{C} = \begin{bmatrix} C_1^J & C_2^J & C_3^J & C_4^J \\ C_1^{X_1} & C_2^{X_1} & C_3^{X_1} & C_4^{X_1} \\ C_1^{X_2} & C_2^{X_2} & C_3^{X_2} & C_4^{X_2} \\ C_1^{X_3} & C_2^{X_3} & C_3^{X_3} & C_4^{X_3} \end{bmatrix}.$$

Suppose that one knows precisely to what extent one wishes to change the flux through the pathway, and the concentrations of the three metabolites, then one may write these relative changes as the vector  $\mathbf{p} = \{\ln(J/J^0), \ln(X_1/X_1^0), \ln(X_2/X_2^0), \ln(X_3/X_3^0)\}$ . The superscript  $^0$  refers to the operating (steady) state. Small changes in  $\mathbf{p}$  can then be approximately written as  $\delta\mathbf{p} = (\delta J/J^0, \delta X_1/X_1^0, \delta X_2/X_2^0, \delta X_3/X_3^0)$ . Similarly one may define the vector  $\mathbf{q}$  to denote the logarithm of the enzyme activities,  $\mathbf{q} = \{\ln(e_1/e_1^0), \ln(e_2/e_2^0), \ln(e_3/e_3^0), \ln(e_4/e_4^0)\}$ . For small desired modulations, one can then estimate the changes in enzyme activities,

$\delta\mathbf{q} = (\delta e_1/e_1^0, \delta e_2/e_2^0, \delta e_3/e_3^0, \delta e_4/e_4^0)$  that are needed to bring about the desired changes in flux and metabolite concentrations. In precise terms:

$$d\mathbf{q}^T = \mathbf{C}^{-1}d\mathbf{p}^T = \mathbf{E} \cdot d\mathbf{p}^T$$

where  $\mathbf{E}$  is the elasticity matrix [similar to the  $\mathbf{M}$  defined in Westerhoff & Kell (1987) and the  $\mathbf{W}$  defined in Westerhoff & Van Dam (1987); see also Sauro *et al.* (1987); generalised in Small & Fell (1989), Hofmeyr *et al.* (1993) and Westerhoff *et al.* (1994)] and where the superscript "T" stands for "transpose". For the linear pathway of Fig. 1(a) the matrix  $\mathbf{E}$  reads as follows:

$$\mathbf{E} = \begin{bmatrix} 1 & -\varepsilon_{X_1}^1 & -\varepsilon_{X_2}^1 & -\varepsilon_{X_3}^1 \\ 1 & -\varepsilon_{X_1}^2 & -\varepsilon_{X_2}^2 & -\varepsilon_{X_3}^2 \\ 1 & -\varepsilon_{X_1}^3 & -\varepsilon_{X_2}^3 & -\varepsilon_{X_3}^3 \\ 1 & -\varepsilon_{X_1}^4 & -\varepsilon_{X_2}^4 & -\varepsilon_{X_3}^4 \end{bmatrix}.$$

One of the special cases discussed above was that of homeostasis where only the flux should be changed at constant concentrations of all metabolites. For this case the vector  $d\mathbf{p}$  reads:  $(dp, 0, 0, 0)$ , such that the recommended modulation of enzyme activities equals  $d\mathbf{p}$  times the first column of  $\mathbf{C}^{-1}$ , i.e., the first column of the matrix  $\mathbf{E}$ . This implies that all enzymes should be activated by the same fraction  $d\mathbf{p}$ . Another special case discussed above is the one where the concentration of  $X_2$  should change by the fraction  $d\mathbf{p}$  at constant concentration of  $X_1$  and  $X_3$  and at constant flux. For this case the vector  $d\mathbf{p}$  reads:  $(0, 0, dp, 0)$  and the requested fractional changes in enzyme activities are given by the third column of matrix  $\mathbf{E}$  multiplied by  $d\mathbf{p}$ . This implies that the enzyme concentrations should be increased in proportion to their elasticity towards  $X_2$ .

The above methodology is exact for small changes. It allows three separate generalisations. First, it can also be calculated which changes in enzyme concentrations are required for any other, predefined but much more complicated, simultaneous change in fluxes and metabolite concentrations. Second, the approach can be generalised to a pathway of any complexity, as both matrix  $\mathbf{C}$  and matrix  $\mathbf{E}$  can be formulated according to a well-defined procedure (Westerhoff *et al.*, 1994; cf., Hofmeyr *et al.*, 1993). And thirdly, the approach can be generalised (in the sense of remaining exact) to finite and large changes in fluxes {and flux ratios; here the corresponding matrix columns need to be integrated}, whenever these are carried out at constant metabolite concentrations (this is because then the elasticity coefficients do not change during the variation).

Educated by Kacser and co-workers (Kacser & Acerenza, 1993; Small & Kacser, 1994), we realise

however, that for planned changes in metabolite concentrations, the approach is also generalizable to large changes, although this then requires the solution of nonlinear equations in sets of unknowns.

### Engineering Control Properties

In the above paragraph we have discussed how fluxes and metabolite concentrations may be engineered. It is also possible to engineer the *control properties* of a pathway. Acerenza (1993) and Westerhoff *et al.* (1994) have dealt with this issue of metabolic design from slightly different points of view. Acerenza asked how one may best design a pathway with desired control properties, whereas Westerhoff *et al.* stressed that from control coefficients one may calculate the corresponding elasticity coefficients. Simpson *et al.* (1995) described two applications in which the control structure of the pathway was altered to increase the production rate of certain amino acids.

### Engineering by Site-directed Mutagenesis

When discussing factors that may influence steady-state fluxes and metabolite concentrations, MCA has tended to focus on factors such as enzyme concentrations and the concentrations of external modifiers (Kacser & Burns, 1973; Kell & Westerhoff, 1985, 1986a,b; Ruijter *et al.*, 1991; Jensen *et al.*, 1993b). The former can be modulated by the use of tuneable promoters under well-defined conditions (Jensen *et al.*, 1993a). The effects of either type of modulation had already been derived by Kacser & Burns (1973). Hofmeyr and colleagues (1986) have analysed the effects of changes in total concentrations of conserved moieties.

In much of genetic engineering, classical point mutations are generated and selected for, or site-directed mutations are made. Can we also implement MCA to predict the effect of such a mutation on steady fluxes and metabolite concentrations? Can we implement such mutations to bring about a very pointed change in a cell's metabolism? Site-directed mutagenesis that leads to changes in  $V_{\max}$  only of the enzyme can be dealt with in virtually the same way as modulation of the amount of enzyme (which has been described by Kacser & Acerenza, 1983; Small & Kacser, 1994) (again, we here remain within the framework of "ideal" metabolism). More often, site-directed mutagenesis aims at changing the binding site of the substrate, or of the transition state complex. Here we shall focus on the former case; a mutation that changes the binding constant,  $K_d$ , or

Michaelis constant,  $K_M$ , of an enzyme for its substrate, for its product or for a modifying metabolite. Previously we have shown that the effect on the steady-state flux of a change in a single  $K_M$  with respect to a metabolite  $X$  (i.e.,  $K_X$ ) of an enzyme  $e_i$  amounts to (Westerhoff & Kell, 1987; Kell & Westerhoff, 1986a):

$$d\ln|J| = -C_i^J \cdot \epsilon_X^i \cdot d\ln(K_X)$$

A metabolite affects a metabolic system through its concentration and corresponding characteristic constants (Michaelis or binding constants), one for each enzyme responding to it. Most importantly, at all positions in any enzyme kinetic rate equation the concentration of any metabolite can be written so as to occur divided by a constant characteristic for the combination of that enzyme and that metabolite. Indeed, this was the basis for the above equation. Accordingly the effect of a change in the concentration of a metabolite on the behaviour of the system can be annihilated by changing all  $K_X$ s by the same factor (but in opposite direction) as that metabolite was changed. Indeed, if all those  $K_X$  values are so changed and the system has unique steady states (Westerhoff & Van Dam, 1987), then the concentration of the metabolite cannot but evolve to that steady-state value. In principle therefore, one can increase the steady-state concentration of any metabolite  $X$  by any factor  $\alpha$  by decreasing through site-directed mutagenesis all  $K_X$ s by that same factor. Then the concentration of all other metabolites will remain the same and so will the fluxes. This then may be the method of choice for bioengineering, because it minimally perturbs the host's own functioning.

When the desired changes are small, then this can be written as:

$$d\ln K_X^i = -d\ln X.$$

This modulation should be effected for all enzymes  $i$  that respond to  $X$ . However, the method is also valid for large changes (with a number of provisions). To double the concentration of  $X_2$  in Fig. 1(a) for instance, at constant concentrations of all other metabolites and at constant flux, one could halve the  $K_S$  of enzyme 3 for  $X_2$  and halve the  $K_P$  of enzyme 2 for  $X_2$ .

This method presupposes that no other parameters are changed by the site-directed mutagenesis. In actual practice it is virtually impossible to achieve this situation completely, but perhaps this need not burden us here too much: in engineering practice it may not be quite essential to have no change at all in the rest of metabolism; it may suffice to minimise such changes. There is however, a more fundamental flaw

with the strategy we proposed in the preceding paragraph: it is impossible only to change the  $K_m$  of one substrate of a reaction. This is because the Haldane relationship requires that the ratio of the Michaelis constants of substrate and product multiplied by the ratio of the reverse and the forward  $V_{max}$  equals the equilibrium constant and the equilibrium constant cannot be changed by site directed mutagenesis or amplification of gene expression (unless the coupling to another reaction is changed). How can this problem be dealt with?

One situation is where only the  $K_m$ s of the reaction for both the substrate and the product are changed by the mutation, without any effect on the  $V_{max}$ s. It is this situation which we shall discuss here; other situations can be analysed as a combination of this effect and an effect on both  $V_{max}$ s. The forward and reverse  $K_m$ s must be changed by the same factor to keep the equilibrium constant constant. For any desired change  $\delta X_2$  in Fig. 1(a), and changes in the  $K_m$ s of enzyme 2 and enzyme 3, we write the changes in the rate of reaction 2:

$$\delta \ln |v_2| = -\epsilon_2^2 \cdot \delta \ln(K_{22}) - \epsilon_1^2 \cdot \delta \ln(K_{21}) + \epsilon_2^3 \cdot \delta \ln(X_2).$$

Equating the change in rate to zero and effecting the equality of the relative changes in Michaelis constants, this prescribes for the change in the  $K_m$ s of enzyme 2:

$$\delta \ln(K_{22}) = \delta \ln(K_{21}) = \delta \ln(X_2)/(1 + \epsilon_1^2/\epsilon_2^2).$$

For the  $K_m$ s of reaction 3 one finds analogously:

$$\delta \ln(K_{32}) = \delta \ln(K_{33}) = \delta \ln(X_2)/(1 + \epsilon_3^3/\epsilon_2^3).$$

These equations are valid for small changes, but the principle can be extended so as to bring about large changes in the concentration of a single metabolite. Again taking  $X_2$  of Fig. 1(a) as an example, one should measure  $X_1$  and  $X_2$  at the initial steady state and avail of the rate equation of enzyme 2. In that rate equation the  $K_m$ s should be multiplied by a factor  $x$  and the desired steady-state concentration of  $X_2$  should be substituted for  $X_2$ . Subsequently one should equate the rate to the original steady-state rate and solve for  $x$ . The  $K_m$ s of the enzyme should then be changed by this factor  $x$ . The same should be done for all enzymes that sense  $X_2$ . In case the characteristic constants for modifiers are affected by the site-directed mutagenesis, the required modulation can be calculated in an analogous way.

† Freely adapted from William James.

## MCA for Everyone?

It is at least arguable (Kell *et al.*, 1989) that the chief reason for the slow take-up of the principles of MCA by metabolic biochemists and biotechnologists lay in the perceived lack of user-friendliness of the jargon and of the mathematical underpinnings of the subject. These have to a large extent been overcome by the development and dissemination of computer programs such as GEPASI (Mendes, 1993, 1996) designed to permit users to input the system of interest and perform MCA and other analyses of the system's properties as its parameters are varied. Information on these and other such programs is available via the World Wide Web at the URL <http://gepasi.dbs.aber.ac.uk/home.htm>.

## Concluding Remarks

We cannot but conclude that the adage that Henrik used to cite may hold: when a thing was new, people said "It is not true". Later, when the truth became obvious, people said "Anyway it is not important", and when its importance could not be denied, people said "Anyway it is not new".†

Yes, it is new, *and* we knew it all along; it's just that Henrik Kacser and the other 'controlniks' had to explain it to the 'refuseniks'.

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