CONTROL ANALYSIS OF MICROBIAL GROWTH AND PRODUCTIVITY

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There is one thing I would be glad to ask you. When a mathematician engaged in investigating physical actions and results has arrived at his conclusions may they not be expressed in common language as fully, clearly and definitely as in mathematical formulae? If so would it not be a great boon to such as I to express them so? – translating them out of their hieroglyphics, that we might also work upon them by experiment. I think it must be so, because I have always found that you could convey to me a perfectly clear idea of your conclusions, which, though they may give me no full understanding of the steps of your process, give me results neither above nor below the truth, and so clear in character that I can think and work from them. If this be possible, would it not be a good thing if mathematicians working on these subjects were to give us the results in this popular, useful, working state, as well as in that which is their own and proper to them?

Letter from Faraday to Maxwell, cited by Schwartz (1986)

INTRODUCTION

Leaving aside downstream processing and the like, it is difficult to imagine an industrial microbiological process in which the maximisation of a metabolic flux is not of primary importance. Now, especially for products of low added value, in which the substrate costs are relatively high, it might be thought that the *yield* (mol product per mol substrate) is what matters most. Yet even here the *flux* of substrate(s) to the product contributes to the *volumetric* productivity.

The fact that actual processes fall short of their theoretical performance stimulates thinking about why they fall short, and how

the process might be modified so as to increase the productivity. In the case of technical aspects, such as aeration and cooling, it is clear that one must determine (from a knowledge of the response of the organism to pO_2 and to temperature) which technical factor is most limiting to a further increase in productivity, and then attempt to improve on that factor.

Not long ago, the foundations were laid for a broadly similar approach to the optimisation of the biological part of the process. Using Metabolic Control Theory (review: Westerhoff & van Dam, 1987), one may determine which biological step(s) most limit the productivity, and then devise the most rational method by which to engineer the microbe's metabolism so as to improve on that step (Kell & Westerhoff, 1986*b*). Because this approach is becoming increasingly widely applied, we here wish to review it in the way in which Faraday (see above) would have wished. This should allow the reader to acquire a sufficient understanding of this approach, without (or before) the necessity of embarking on the relevant calculations. Although the Metabolic Control Theory has tended to focus on the optimisation of metabolic fluxes, it can equally well be applied to the maximisation of any other metabolic variable which may be relevant to the productivity.

Of course, traditional and empirical methods of mutation and strain selection have led to the development of production strains for (say) antibiotics which outstrip the starting isolates by orders of magnitude in terms of their ability to produce the desired end-product (Calam, 1987). Thus, the penicillin fermentation has, through countless rounds of mutation and selection, increased its final titre from some 2 Units/ml to some 50 000 Units/ml over the last 40 years or so; however, as Cooney (1979) pointed out, such a strain still only converts some 6% of the added carbon source to the desired product. By contrast, the yield in say the glutamate fermentation may be 50% or more (in terms of added carbohydrate, e.g. Meers & Milsom, 1987); here the improvements to be desired are generally taken to relate more to strain stability, resistance to bacteriophage and in fermentor control algorithms. In both (and indeed all) cases, however, and certainly from an economic standpoint, it is the *volumetric* productivity which should be maximised, i.e. the *flux* of substrate to product (per unit fermentor volume or per unit produced). Our aim is to give an overview of certain more-or-less process-independent means by which one might seek to engineer any of these improvements.

The crux of the argument in what follows may therefore be summarised:

(1) The yields of even well-studied and 'mature' fermentations are significantly lower than those theoretically possible. One may imagine that the evolution of such strains has become 'stuck' in a region of 'evolutionary space' from which *random* mutation and selection is unlikely to permit its escape, or the evolutionary pressure on these strains has not been towards a metabolic structure that is optimal for our purposes. 'Novel' strategies are therefore necessary for the improvement of such processes;

(2) If the novel strategies are to be rationally based, the *conditio sine qua non* is an improved understanding of the relevant metabolic pathways, and their control, in microorganisms in general and in producer strains in particular;

(3) Given the modern availability of powerful genetic and protein engineering techniques, any data available in (2) may be used *rationally* to choose which protein(s) to clone and/or to modify for the desired purpose(s);

(4) A re-emergent formalism (which we review), known as the Metabolic Control Theory (MCT), allows one *rigorously* to define the control structure of biochemical pathways of arbitrary complexity under almost all conditions; especially with the advent of cheap microcomputers, it constitutes a subset of the Universe of available modelling approaches, at an appropriate level of complexity, and is proposed as a formalism of choice for work in this area. We give examples of its successful use and (*a posteriori*) its predictive power. Whilst much of the MCT approach, with the wisdom of hindsight, is only applied common sense, its benefit is that it does provide a correct, satisfying and generally applicable formalism for the appropriate description of metabolic processes. Even where technical difficulties proscribe the *rigorous* application of the MCT, approximate analyses carried out within its framework can give an improved understanding of the control and 'engineerability' of a metabolic pathway.

(5) Especially because MCT describes only the control *structure*, and not absolute fluxes, other approaches must be used to supplement the information available using MCT; attention is drawn to some of these, such as Biochemical Systems Theory and Mosaic Non-equilibrium Thermodynamics.

We begin with an outline survey of the Metabolic Control Theory,

first in an historical context and then in terms of its chief tenets and theorems.

METABOLIC CONTROL THEORY

Historical overview

The formalism which we refer to as the MCT is usually taken to originate, so far as biochemistry and microbiology are concerned, with the work of Kacser & Burns (1973) in Edinburgh and of Heinrich & Rapoport (1973, 1974) in Berlin. It followed and paralleled similar contributions from Higgins (1965) and Savageau (1972, 1976). In fact the generalised approach had been known in economics and many other fields for some time previously (Cruz, 1973), since it may be applied to any system in which 'users' (people, enzymes) interact with each other by means of diffusible intermediates (money, metabolites) which change their nature (or value) concomitant with each interaction, and at rates which depend upon the concentrations of all other intermediates in the system. In other words, it provides a means of dealing with networks in general and with metabolic networks in particular.

For a variety of reasons, probably owing more to sociology than to science, the MCT was little used during the 1970s by other than its originators (see Rapoport *et al.*, 1976; Heinrich *et al.*, 1977, Kacser & Burns, 1979). However, perhaps because of its success in resolving important controversies in heterokaryon genetics (Kacser & Burns, 1981) and mitochondrial oxidative phosphorylation (Groen *et al.*, 1982*a*, Wanders *et al.*, 1984*a*; Westerhoff *et al.*, 1987*a*, Westerhoff & van Dam, 1987), and the establishment of a unified terminology (Burns *et al.*, 1985), as well as being reinvented for enzymes (Ray, 1983), it has been reviewed (Groen *et al.*, 1982*b*; Westerhoff *et al.*, 1982, 1984*a*; Derr, 1985, 1986; Kell & Westerhoff, 1985, 1986*a*, *b*; Porteous, 1985; Hofmeyr, 1986; Brand & Murphy, 1987; Kacser & Porteous, 1987; Kell, 1987; Nimmo & Cohen, 1987; Westerhoff & van Dam, 1987; Kacser, 1988), extended (Stucki, 1983; Westerhoff & Chen, 1984; Fell & Sauro, 1985; Chen & Westerhoff, 1986; Keleti & Vértessy, 1986; Welch *et al.*, 1988; Hofmeyr *et al.*, 1986; Sorribas & Bartrons, 1986; Westerhoff & Kell, 1987, 1988; Sauro *et al.*, 1987; Westerhoff & van Dam, 1987; Westerhoff *et al.*, 1987*b*) and adopted (Gellerich *et al.*, 1983; Doussière *et al.*, 1984; Westerhoff & Arents,

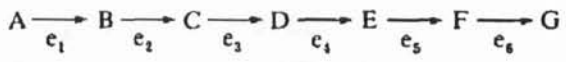


Fig. 1. A typical linear (unbranched) metabolic pathway, in which an initial substrate A is converted by enzyme-catalysed reactions via a series of metabolic intermediates (B, C, D, E, F) into a product G. Under the usual conditions considered, [A] is maintained constant, or at a value far above the $K_{m,\,app}$ of the first enzyme in the pathway and the activation or inhibition constants for the other enzymes in the pathway. The flux to G (J_G) and the concentrations of the metabolic intermediates are then variables, which attain appropriate values when the system relaxes to a steady state and which depend upon parameters such as the enzyme concentrations and catalytic constants, temperature, pH and the concentration of external modifiers.

1984; Wanders *et al.*, 1984*b*; Walsh & Koshland, 1985; Mazat *et al.*, 1986; Groen *et al.*, 1986; Salter *et al.*, 1986; Dixon *et al.*, 1987) in an increasing number of quarters. At the time of writing (spring 1988), it has not been greatly exploited (in published form) for biotechnological processes, although we are aware of a number of groups who *are* now making use of MCT to such ends. It is in the hope of extending the user base still further that we again review it for the present audience. Because the chief difficulty for the user probably lies in becoming acquainted with the terminology of the formalism, we lay special emphasis on the distinction between the two main coefficients used: *control coefficients* and *elasticity coefficients*. This review will go into a depth sufficient to permit the reader to acquire a working understanding of the MCT, whilst those for whom our discussion may seem superficial are referred to a recent extended analysis (Westerhoff & van Dam, 1987). Although some of the terms now used differ from those in the early literature, we adopt throughout the unified terminology agreed by the originators of MCT (Burns *et al.*, 1985).

The idealised metabolic pathway

It is convenient to consider a linear (unbranched) metabolic pathway (A . . . → . . . G) (Fig. 1), in which a 'starting' substrate A is converted via a series of intermediates to a product G, with each reaction being catalysed by an enzyme (e_1, e_2, e_n, etc.), each present at a concentration e_n (we thus drop square brackets for convenience). Under steady-state conditions, the flux J through the pathway (in say nmol (min mg protein)$^{-1}$) is obviously equal to dG/dt (if there is no sink for the product) and the concentrations of intermediates are steady in time. As is usual, we consider that A is present at a concentration sufficient for its effect upon the reaction rates to remain constant during the time of observation. This condition is

met if [A] is far above the K_m (K_i) value(s) of the enzymes with respect to A, if some other process produces A at the same rate at which it is consumed, or if the concentration of A is strongly buffered. In the same sense, we assume that the increase in G with time is sufficiently small that J remains constant. Of course there is a certain arbitrariness in this definition of a steady state, but, since we wish to relate the theory to actual experiments, the operational definition is appropriate. The effective constancy of A and G is essential for the validity of the mathematical component of the MCT. If A and G do vary, then an extended pathway should be considered (see below). It is not essential that the pathway is linear. Moreover, all reactions may be reversible and be influenced by any other metabolite.

In the MCT (and indeed generally) it is crucial to distinguish between parameters and variables: *parameters* are properties of the experimental system set either by the experimenter (e.g. temperature, pH) or by nature (K_m of enzyme i, k_{cat} or V_{max} of enzyme i) that remain constant during an experiment, whilst the latter (such as the concentration of intermediary metabolite X, or the flux J) only attain a constant value during a steady state; the steady-state magnitude of any variable is set by those of all the parameters.

Flux-control coefficients

Although we shall see that this question is incorrect in principle, the next question which is usually asked is, 'which enzyme is catalysing the rate-limiting step?' Since we have a (steady-state) situation in which all reactions are proceeding at the same rate, we cannot discern the extent to which a particular enzyme is controlling a flux merely by simple observation of that flux. Let us perform a *Gedanken* experiment with enzyme e_4 of our prototypical metabolic pathway of Fig. 1. We know that if we remove it completely the flux will drop to zero (this is one criterion that the enzyme is actually *in* the pathway), and if we raise its concentration to an arbitrarily high value the flux will reach a plateau where it is 'limited' by the activities of the other enzymes in the pathway. At least three simple possibilities for describing the relation between J and e_4 then exist (Fig. 2). In case (a), doubling (the concentration of) e_4 has a negligible effect upon the overall flux; we would be justified in stating that e_4 exerted no flux control. In case (b), doubling e_4 doubles J; we would (in fact) be justified in stating that e_4 was completely flux-

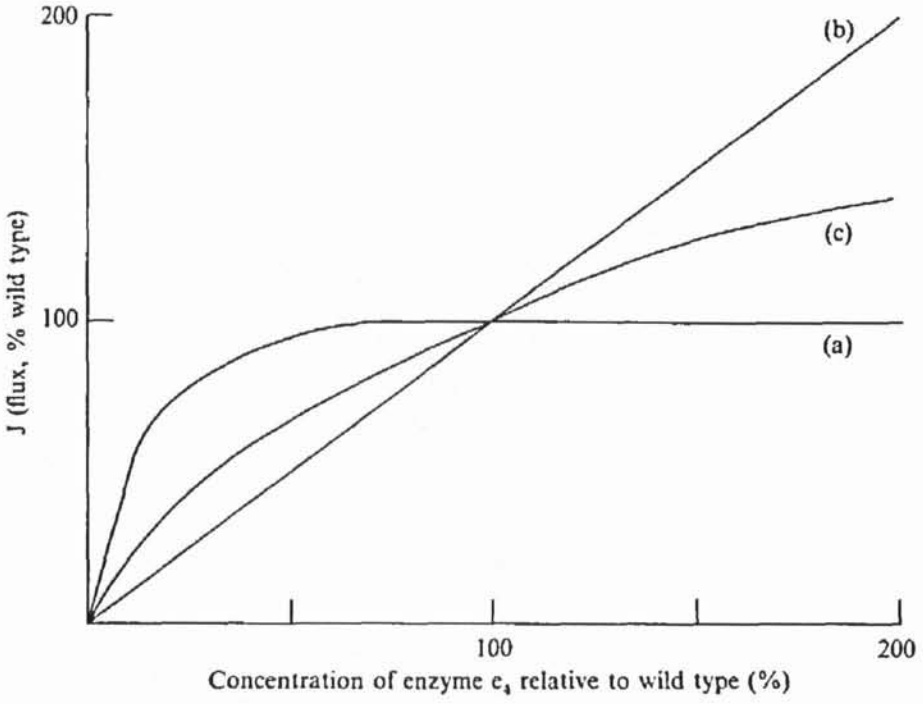


Fig. 2. Possible effects of the concentration of any enzyme such as e_4, modified for instance by genetic means, on the flux through a pathway such as that of Fig. 1 in which it is a catalyst. For further details, see the text. From Kell & Westerhoff (1986*b*) © Elsevier Publications. Reprinted with permission.

controlling. Case (c), however, would present us with something of a quandary, in that doubling e_4 causes an increase, but not a proportional increase, in J; clearly e_4 is not rate-limiting but it is not not-rate-limiting either, since it does in some way contribute to the control of the flux. A two-valued logic system of rate-limiting *vs.* non-rate-limiting is inappropriate for our purposes, and Higgins (1965), Kacser & Burns (1973, 1979) and Heinrich & Rapoport (1974) introduced the *flux-control coefficient* C^J_e ($e = e_n$) to express *in quantitative terms* the extent to which an enzyme in (or affecting) a metabolic pathway was controlling the pathway flux J. Since (Fig. 3) the ratio between the change in flux ΔJ occasioned by a change in enzyme concentration Δe itself depends both upon the magnitude of Δe and the absolute magnitude e, $\Delta J/\Delta E$ may not be the best measure of flux control. Rather, the flux-control coefficient is made dimensionless; it is defined as $[(dJ/J)/(de/e)]_{ss}$, where the subscript ss indicates that measurements are made under steady-state conditions. That is, after changes are made in e, one considers the difference between the original steady state and the new steady state. The measurement must also be made under conditions in which all parameters other than the concentration of e are held constant.

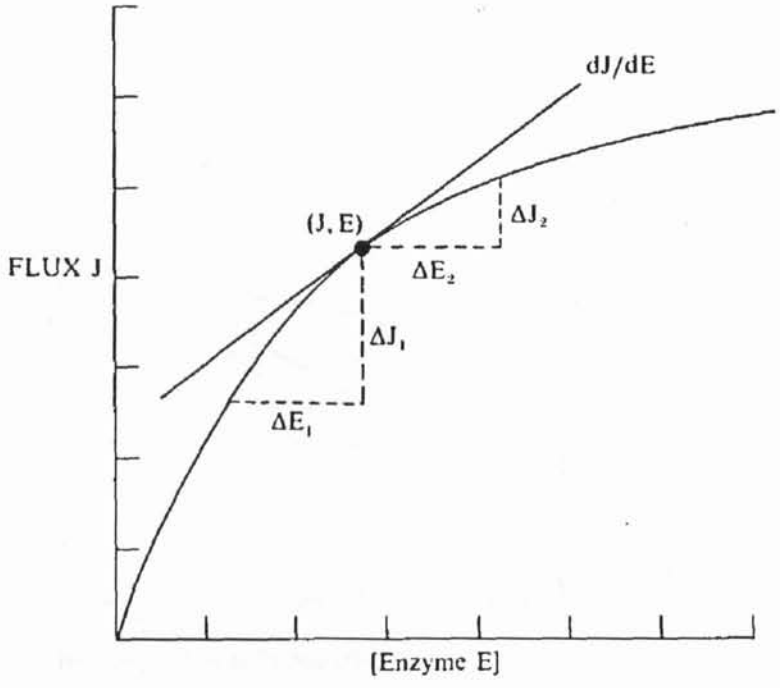


Fig. 3. The flux-control coefficient of an enzyme E. If the concentration of enzyme E in a metabolic pathway is changed by an amount ΔE, then the pathway flux will change by an amount ΔJ. However, because of the probably non-linear relationship between J and E, $\Delta J/\Delta E$ depends both on the value of (J, E) and on the magnitude (and sign) of ΔE. One therefore defines (Higgins, 1965) the flux-control coefficient using limiting changes and in dimensionless terms, as $[(dJ/J)/(dE/E)]$.

Thus the flux-control coefficient is defined as the *fractional* change in pathway flux divided by the fractional change in enzyme concentration (or activity) when these changes are made (infinitesimally) small. Since dJ/J is mathematically equivalent to $d\ln J$, the flux-control coefficient may be obtained as the slope of a plot of *J versus* e on log–log paper, at the value of (J, e) prevailing. As in Fig. 2, the flux-control coefficient will most often take a value between 0 and 1, although negative values (and values > 1) are possible. Thus, we may reiterate, the flux-control coefficient provides a quantitative expression of the magnitude of the flux control exerted by an enzyme on the flux through a metabolic pathway.

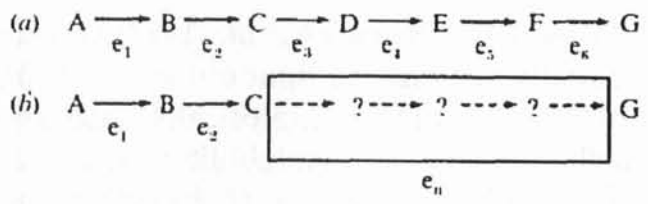
The flux-control summation theorem

Evidently, by means similar to that which we used for e_4 in our *Gedanken* experiment above, we may determine the flux-control coefficient of *each* enzyme in the pathway, without for the moment worrying about *why* they may take the values they possess. What use is this to us? It turns out, and has been proven rigorously (Kacser

& Burns, 1973; Heinrich *et al.*, 1977; Westerhoff & van Dam, 1987), essentially because we limit ourselves to 'infinitesimal' changes and transitions between asymptotically stable steady states (and provided that the intermediate metabolites including any allosteric modifiers exhibit pool behaviour), that *the sum of the flux-control coefficients of the enzymes in a metabolic pathway (or interacting with it via allosteric modifiers) equals unity*. This is known as the *flux-control summation theorem*. This then gives an exact framework for what is intuitively clear, if not always implemented in discussions of metabolic control: the flux control is distributed over all the enzymes in a metabolic network. Rather than asking the question 'which enzyme controls the flux in a metabolic pathway?' a more useful question is then 'how is flux control distributed between the enzymes in the network?' Thus a knowledge of the flux-control coefficients of the enzymes of interest permits one *rationally* to choose which enzymes one might best seek to clone and/or to modify to increase the flux of interest.

A rather obvious corollary of the flux-control summation theorem is that, if a linear metabolic pathway contains n enzyme types, then the *average* value of the flux-control coefficient will be $1/n$, so that in long pathways the flux-control coefficient of any enzyme is likely to be small. This not only accounts for the prevalence of genetic dominance in heterokaryons (Kacser & Burns, 1981) but provides a satisfying intellectual explanation of *why* so many rounds of mutation and selection are required to obtain a more productive strain, since flux-control is constantly shifting from one enzyme to another as the strain-improvement programme progresses. By contrast, individual enzymes are liable to exert a very strong flux control in short pathways; the control by inosine monophosphate dehydrogenase on the flux to product in commercial guanosine fermentations appears to be a case in point (Miyagawa *et al.*, 1985).

From the point of view of the production of say antibiotics, or of other secondary metabolites, where the exact metabolic pathways are often still not known precisely, a black-box approach (Fig. 4) to the estimation of flux-control coefficients is permissible (and probably preferable). Provided that one may induce the coordinate induction or repression of a group of enzymes, as seems to be the case with some but not all Streptomycetes (Malpartida & Hopwood, 1984; Chater & Bruton, 1985; Stanzak *et al.*, 1986), such groups of enzymes may be considered as a single enzyme for the purpose of estimating their contribution to flux control (Kacser, 1983;



(a): $\Sigma C^J_{e_i} = 1$

(b): $C^J_{e_1} + C^J_{e_2} + C^J_{e_n} = 1$

Fig. 4. Estimation of the contribution of a particular *segment* of metabolism to the control of the flux through the whole pathway does not require that we know the identity of each of the enzymes concerned. This is of course a fairly common circumstance in studies of secondary metabolism. The flux control coefficient of a *segment* is the sum of the flux-control coefficients of each of its constituent enzymes. In other words, $C^J_n + C^J_{n+1} + C^J_{n+2} = C^J_{(n+(n+1)+(n+2))}$

Hofmeyr *et al.*, 1986; Westerhoff *et al.*, 1987*a*). Of course, by their very nature, as compounds not necessary for cellular growth and typically produced during idiophase in batch cultures, we may expect that it is only *during* such an idiophase that the enzymes of secondary metabolism actually appear. This would mean that it is the activities of the enzymes of secondary metabolism *per se*, and not of those of primary metabolism that are necessary to produce the carbon skeletons that form the substrates for secondary metabolism, that exert most flux control on the rate of secondary metabolite production. This suggestion is indeed borne out in practice (Běhal, 1986), and suggests (at least for these cases) that 'rational' cloning approaches should therefore concentrate mainly upon the enzymes of secondary metabolism themselves (see Fig. 5).

Control coefficients for pathway substrates, external effectors and metabolite concentrations

In contrast to some suggestions (Meiske & Reich, 1987), metabolic control theory is not limited to the control by parameters (such as enzyme activities) that linearly affect reaction rates. Indeed (Kacser & Burns, 1973), we may define flux-control coefficients for the pathway substrate and for any other external effector (e.g. an inhibitor), exactly as we did above for the enzymes of the pathway, in terms of the fractional change in flux divided by the fractional change in effector (concentration). Thus for an external effector I, $C^J_I = [(\mathrm{d} \ln J)/(\mathrm{d} \ln \mathrm{I})]_{ss}$, under conditions in which no other changes are made to the system other than I, C^J_I may take any value (positive, negative, >1, etc.).

In the steady state, the concentration of intermediary metabolites

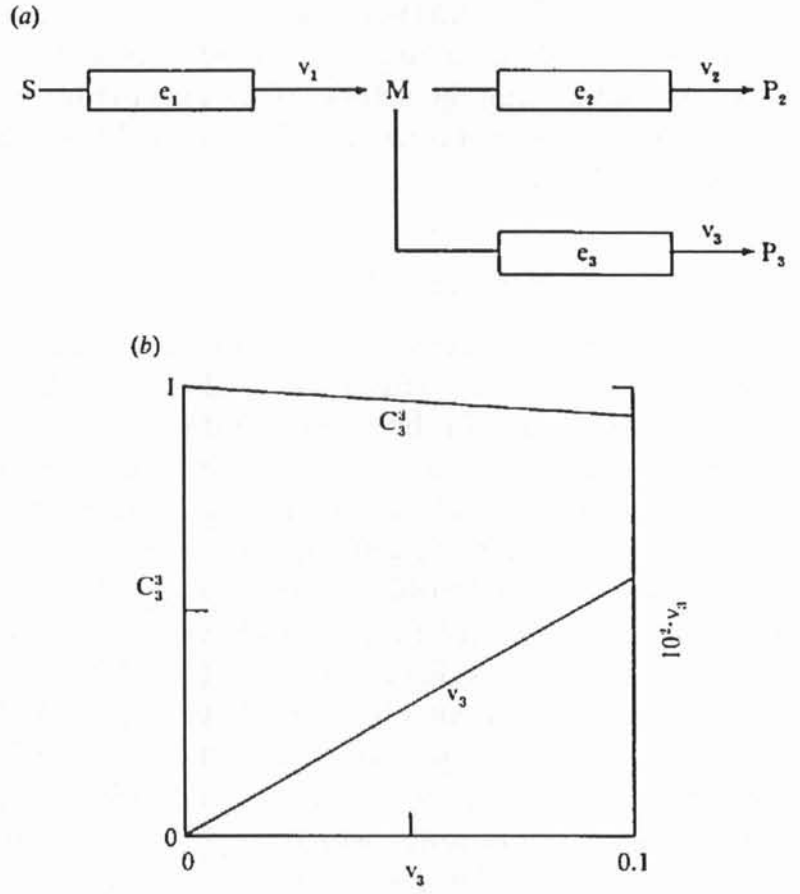


Fig. 5. Enzymes of secondary metabolism, with low values of k_{cat} or V_{max}, tend to have a high flux-control coefficient for the flux to the secondary metabolite. (a) The pathways modelled, in which P_3 is the secondary metabolite produced from an intermediate M, which in the absence of e_3 is part of another pathway (producing P_2). (b) The flux-control coefficient (upper full line) and flux through secondary metabolism (lower full line) are given as a function of the V_{max} of secondary metabolism. The plots were calculated using the (normalised) rate equations: $v_1 = 1/(1 + M)$; $v_2 = M$; $v_3 = V_3.M$ and the steady-state condition $v_1 = v_2 + v_3$. The flux-control coefficient C^3_3 was evaluated using Eq. 4.115 of Westerhoff & van Dam (1987). The fraction of the pathway flux flowing through secondary metabolism = $V_3/(1 + V_3)$.

is unchanging, and is obviously determined by the enzymes of the pathway (and of those interacting with it via the production or utilisation of allosteric effectors). The concentration-control coefficient $C^x_{e_i}$ for enzyme e_i *on* metabolite X is $[(d \ln X)/(d \ln e_i)]_{ss}$, and there is a family of concentration control coefficients for each metabolite, one for each enzyme in the pathway or to which the pathway is connected via diffusible intermediates. There is also a summation theorem for the metabolite concentration-control coefficients: $\Sigma C^x_{e_i} = 0$, or in other words the sum of the concentration-control coefficients for a set of enzymes on metabolite X is zero (Heinrich

et al., 1977). This means that some metabolite concentration-control coefficients will have negative values, as one would expect; in general, stimulating the activity of an enzyme for which the metabolite of interest is a *substrate* will tend to lower the concentration of that metabolite.

Yield and efficiency control coefficients

Traditionally, MCT only discusses the control of fluxes and of metabolite concentrations. However, it also sheds light on the control of any variable within the system under consideration. Thus, MCT has discussed proton-linked free energy transduction in terms of the control of proton electrochemical potential differences and phosphate potentials by the participating enzymes (Westerhoff & van Dam, 1987). For microbial biotechnology, MCT may be extended to include the control of yield (which is the control of a ratio of fluxes (Westerhoff & Kell, 1987)) or the control of the (non-equilibrium) thermodynamic efficiency of the process of interest. Thus, depending upon the purposes for which one wishes to improve a microbial strain (or, indeed, process), one should focus on the appropriate control coefficients, which may be for output flux, metabolite concentration, yield or efficiency.

Elasticity coefficients

Thus far, we have ignored the fact that the *turnover number* of an enzyme *in situ* is of course a function of the concentrations of its substrate(s), product(s), allosteric modifiers and so on. MCT refers to such properties in terms of the *elasticity coefficients*, which are defined, in a way that is mathematically similar to the control coefficients, as the fractional change in the turnover number of a particular enzyme divided by the fractional change in concentration of the effector of interest, under conditions in which all other parameters *and variables* are held constant. Thus the elasticity coefficient $\varepsilon_X^{e_i}$ of enzyme e_i towards metabolite X is $= \partial \ln v_i / \partial \ln X$. In other words, the elasticity coefficient may be derived from the relevant enzymatic rate equation simply by taking the (partial) derivative thereof, under conditions in which all other modifiers are present at their *in vivo* values.

A convenient way to remember the shorthand embodied in MCT is to note that in the definitions of flux-control and elasticity coeffi-

cients, the subscript represents the *cause* and the superscript the *effect* of a particular change. It may be noted that the elasticity coefficient is the property that an enzymologist would measure while studying a single enzyme; he would determine the dependence of the reaction rate on the concentration of substrate(s) at constant and defined concentrations of product(s) and modifiers. In a double logarithmic plot of this dependence, the slope would amount to the elasticity coefficient of the enzyme towards its substrate under the assay conditions. Note also that the elasticity coefficients differ from the control coefficients in that when the latter are measured (a) only the parameters are held constant and (b) the system is allowed to relax to a new steady state. In addition, the elasticity coefficients are *local* (reflecting the effects of single effectors on the rates of single enzymes), and are thus *partial* derivatives, whereas the control coefficients are *global* (reflecting the effects of single enzymes on *mixtures of enzymes* causing a flux through a metabolic pathway). Control coefficients amount to what a cell biologist would measure, i.e. the effect of a change in a metabolically relevant parameter after allowing the cell to adjust itself to the change and to attain a new steady state.

We would stress that, despite an apparently widespread view to the contrary, *intermediary metabolite concentrations do not control fluxes*; it is the *enzymes* and their properties *themselves* which *control the fluxes* – intermediary metabolite and effector concentrations adjust themselves to their steady values as a *consequence* of these. Thus 'explanations' such as 'the flux through pathway X is low because the concentration of metabolite M is significantly below the K_m of enzyme Y' are insufficient, and should be avoided. However, since the global behaviour of a system is related to the behaviour of its individual components, we may expect that the flux- (and metabolite concentration-)control coefficients are related in some way to the elasticity coefficients. This is indeed the case.

The connectivity theorems

If one inhibits a particular enzyme (using a specific, irreversible or non-competitive inhibitor) in a pathway through which metabolites are being transformed at a flux J, the first effect will tend to be the transient build-up of the enzyme's substrate. If our (Michaelis–Menten) enzyme had been operating far above its K_m, its turnover would thereby be increased but little to accommodate the block

and the flux would be modified nearly in proportion to the decrease in the activity of the enzyme. From what we have learned above, this would be an enzyme with a low elasticity (little change in turnover for a significant change in substrate concentration) and a high flux-control coefficient (large change in flux for a given change in enzyme concentration/activity). It is evident that the converse would also have been true for an enzyme operating well below its K_m. These qualitative statements are embodied in the *flux-control connectivity theorem* which states 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* (Kacser & Burns, 1973) or, in our shorthand: $\Sigma C^J_{e_i} \cdot \varepsilon^{v_i}_x = 0$. Matrix methods allow a convenient determination of the flux-control coefficients *from the elasticities alone* (Fell & Sauro, 1985).

A second connectivity theorem (Westerhoff & Chen, 1984) serves to express metabolite concentration-control coefficients in terms of elasticities (Westerhoff *et al.*, 1984; Sauro *et al.*, 1987; Westerhoff & Kell, 1987; Westerhoff & van Dam, 1987). In addition, for a specific external inhibitor acting upon an enzyme e_i, $C^J_I/I = (C^J_{e_i} \cdot \varepsilon^{e_i}_I)/I$, in other words the flux-control coefficient of an inhibitor = the flux-control coefficient of its target enzyme times the elasticity of the enzyme towards the inhibitor. The division by I ensures the definability at $I = 0$, the physiological concentration (Westerhoff & van Dam, 1987). For a 'perfect', tight-binding inhibitor (i.e. where one molecule of inhibitor completely inhibits one molecule of enzyme) $\varepsilon^{e_i}_I = -I/e_i$, such that $C^J_{e_i} = -\mathrm{d}\ ln\ J/\mathrm{d}\ ln\ (I/e_i)$, i.e. the flux-control coefficient equals the percentage decrease in the steady-state flux caused by an inhibition of 1% of the target enzyme. This provides a convenient means for the estimation of the flux-control coefficients from suitable inhibitor titrations (Groen *et al.*, 1982; Westerhoff *et al.*, 1984; Kell & Westerhoff, 1986*a*, *b*; Westerhoff & Kell, 1988), since the amount of enzyme is obtained by extrapolation of the titration curve to the abscissa, the fractional change therein by the slope of the line joining this point with the starting point of the titration, and the fractional change in flux is the initial slope of the titration curve. Thus the flux-control coefficient falls out directly as the ratio of two normalised slopes (Fig. 6).

In sum, the flux-control coefficients tell us quantitatively *which* enzymic steps control metabolic fluxes of interest, whilst the elasticities tell us which interactions or properties of the enzymes underlie the systemic observables. The connectivity and summation proper-

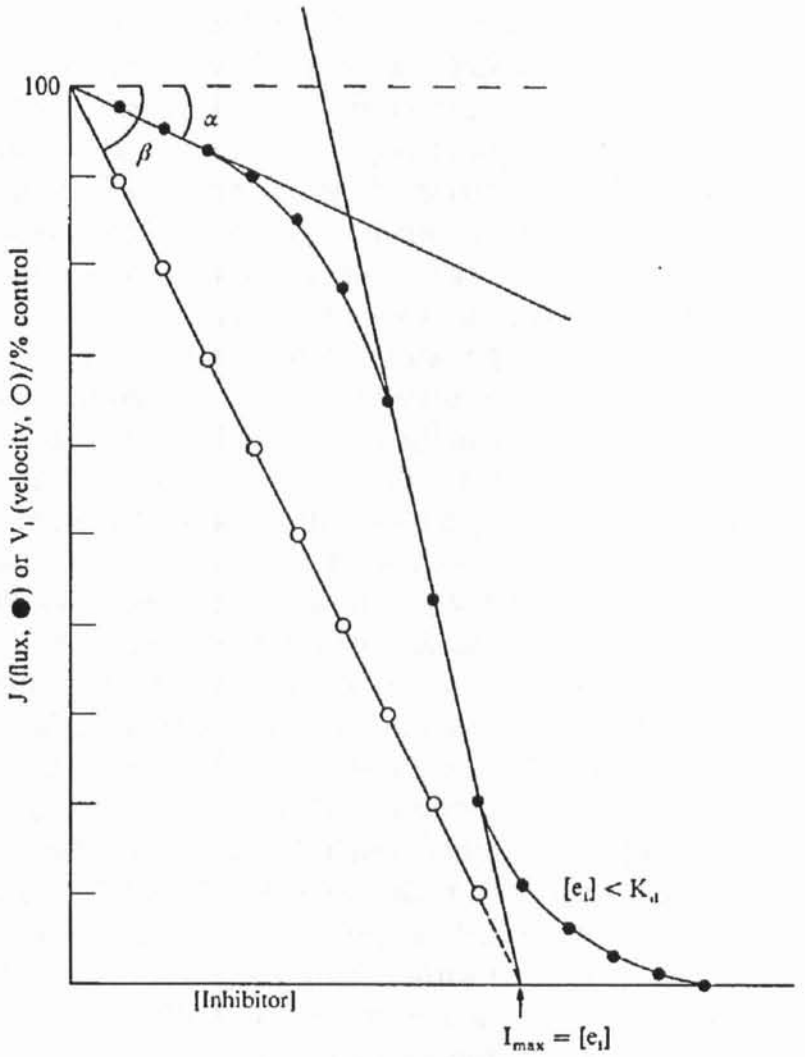


Fig. 6. Estimation of the flux-control coefficient $C^J_{e_i}$ of an enzyme e_i by the use of a tightly-binding and specific inhibitor of that enzyme. Here, both the normalised pathway flux J (●) and the normalised velocity v_i of the (functionally) isolated enzyme (○) are titrated using a tight-binding, specific and stoichiometric inhibitor of the enzyme. Allowing for the fact that the dissociation of the enzyme–inhibitor complex at very low enzyme concentrations must be taken into account, the initial concentration of enzyme = the extrapolated concentration of inhibitor from the titration curve itself. The flux-control coefficient of the enzyme is given by tan α/tan β. Note that the experimental curve for the functionally isolated enzyme would also show a tail, which is omitted here to indicate the extrapolation procedure to be used. From Kell & Westerhoff (1986*b*) © Elsevier Publications. Reprinted with permission.

ties describe how these enzymic properties determine the distribution of control between the enzymes.

More complicated pathways

Although the MCT is becoming relatively 'mature', additional theorems and derivations are still being obtained, as an increasing body

of workers becomes interested in the use of the MCT. In particular, branched pathways (Kacser, 1983; Westerhoff & Arents, 1984; Fell & Sauro, 1985; Sauro *et al.*, 1987; Kacser, 1988), conserved cycles of cofactors and futile cycles (Hofmeyr *et al.*, 1986; Sorribas & Bartrons, 1986; Westerhoff & van Dam, 1987) have been dealt with. The existence of a branched pathway adds two major problems to the analysis. First, the number of enzymes now exceeds the number of metabolites, since two enzymes act on (i.e. use as a substrate) the metabolite appearing at the branch point, so that additional theorems are necessary to express the control coefficients simply in terms of the elasticities. In fact, the minimum and sufficient piece of knowledge that is additionally necessary is the flux ratio at the branch-point. From a biotechnological point of view, and as follows from the opening statement, enzymes in the other branch(es) of a branched pathway will tend to have *negative* flux-control coefficients for the production of the metabolite of interest. Thus, as is of course already practised, maximisation of the flux of interest generally requires the inhibition of competing fluxes.

A particularly interesting case (Welch *et al.*, 1988; Westerhoff & Kell, 1988), and one whose frequency of occurrence is becoming increasingly apparent (Welch & Clegg, 1986; Srere, 1987; Srivastava & Bernhard, 1987), is that in which metabolites are not freely diffusible (i.e. organised as 'pools'), but are passed directly from one enzyme to the next without being released into the 'bulk' intracellular medium. In fact a number of secondary metabolic systems also appear to exploit this organisational principle (Běhal, 1986). In this case, the flux-control summation theorem is violated when judged by the use of specific inhibitors (since inhibiting *any* enzyme in such an 'organised' pathway inhibits the flux in direct proportion, and this is true for each enzyme in the pathway). Such a finding provides a rigorous means of detecting the existence of such 'microcompartmentation' (Kell & Westerhoff, 1985; Westerhoff & van Dam, 1987; Westerhoff & Kell, 1988).

EXPERIMENTAL APPROACHES

The experimental assessment of the magnitude of flux-control coefficients follows more-or-less directly from their definition: one studies the change in flux engendered by a change in the activity or concentration of the enzyme of interest. One approach was given above, viz. the use of specific inhibitors to modulate an enzyme's activity.

Using reconstituted systems of soluble and non-interacting enzymes *in vitro*, the flux-control coefficients may be simply evaluated by titrating in the enzyme of interest (Torres *et al.*, 1986). Alternatively, the activity of a particular enzyme may be changed by genetic means. In eukaryotes, one may create heterokaryons which express different amounts of the enzyme of interest, and Kacser and colleagues have used this approach with *Neurospora crassa* (Flint *et al.*, 1980, 1981) and *Drosophila melanogaster* (Middleton & Kacser, 1983). It was found that, since the flux-control coefficient of most enzymes is low, decreasing their concentration by even 50% often has only a modest effect upon the pathway flux; thus it is to be expected, and is found, that most mutations in diploid organisms are recessive (Kacser & Burns, 1981).

Dean and colleagues (Dykhuizen *et al.*, 1987) used a series of mutants with altered activities of the *lac* permease or β-galactosidase to determine the control coefficients of these enzymes with respect to the 'fitness' (growth rate) of *E. coli* in lactose-limited chemostat cultures, finding values of 0.6 and 0.02 respectively. Walter *et al.* (1987) also found a fairly high flux-control coefficient for the glucose–PTS system on the rate of glycolysis in washed cells of *Clostridium pasteurianum*, by exploiting the ability of a non-specific but non-permeant inhibitor selectively to inhibit the glucose–PTS. In haploid organisms, and microorganisms generally, it is nowadays easier to assess the flux-control coefficient of an enzyme by placing its expression under the control of a promoter of variable strength. Assuming that pleiotropic effects are absent, only the concentration of the 'target' enzyme should be modulated by varying the promoter strength. This elegant approach was pioneered by Walsh & Koshland (1985), who placed citrate synthase in *E. coli* under the control of the *tac* promoter, a hybrid composed of parts of the *trp* and *lac* promoters and whose strength (under conditions in which the *lac* repressor is present) is a function of the concentration of isopropyl β-D-thiogalactopyranoside (IPTG). It was found (Walsh & Koshland, 1985) that the flux-control coefficient of citrate synthase was variable but generally low on a glucose-plus-acetate medium, whilst on an acetate medium alone the flux-control coefficient was high (but not unity). Clearly this is an exceptionally powerful approach to the understanding of metabolic regulation *in vivo*, and Nimmo & Cohen (1987) review other possibilities. For instance, the phosphofructokinase (PFK) reaction is thought to be one of the most flux-controlling steps in glycolysis in many organisms (Hess &

Boiteux, 1971). However, a recent study by Heinisch (1986), who cloned the structural genes for yeast PFK and found that the rate of ethanol production was essentially unchanged even when their expression was increased 3.5-fold, illustrates both that strong flux control is not associated with this reaction and indeed how useful this general approach can be in solving problems of metabolic control. In this regard, we might also mention that analogous principles might be used to estimate the contribution of different organisms to the control of flux through mixed cultures or consortia.

The experimental assessment of elasticity coefficients is less highly developed than is that of the flux-control coefficients, since it is in general technically difficult *in vivo* to change but one concentration of a metabolite whilst holding all the others constant. Of course this is far simpler *in vitro* (Torres *et al.*, 1986), and the development of cell-free systems capable of secondary metabolite production (Suckling, 1984; Běhal, 1985) may be expected to lead to a much clearer understanding of the control structure of such pathways. Perhaps the easiest approach for assessing elasticities (Groen *et al.*, 1986) is to calculate them simply from the enzyme kinetic parameters and the measured concentrations of substrates, products and known modifiers. Potential differences between *in vitro* and *in situ* conditions generate uncertainties in this approach. Another promising *in vitro* approach perturbs flux through a pathway in two different ways and measures the change in both the reaction rate (flux) and substrate and product concentrations of the enzyme of interest. This is suitable if the elasticities of the enzyme of interest towards other modifiers are zero. Finally, elasticities may be determined if it is possible to manipulate the pathway flux in two independent ways under conditions in which the concentration of intermediate metabolites can be monitored (Kacser & Burns, 1979).

SOME EXAMPLES OF WHAT CONTROL ANALYSIS CAN TELL US

At the simplest level (Westerhoff *et al.*, 1982; Kell, 1987), given the usual distinction between reactions which generate and which consume ATP (Fig. 7), it is legitimate to ask the question, 'which contributes more to the control of microbial growth rate in unrestricted batch culture – catabolism or anabolism?' Previous studies, whilst assuming that a unique answer was possible, have given conflicting answers to this question for *E. coli* growing in glucose minimal

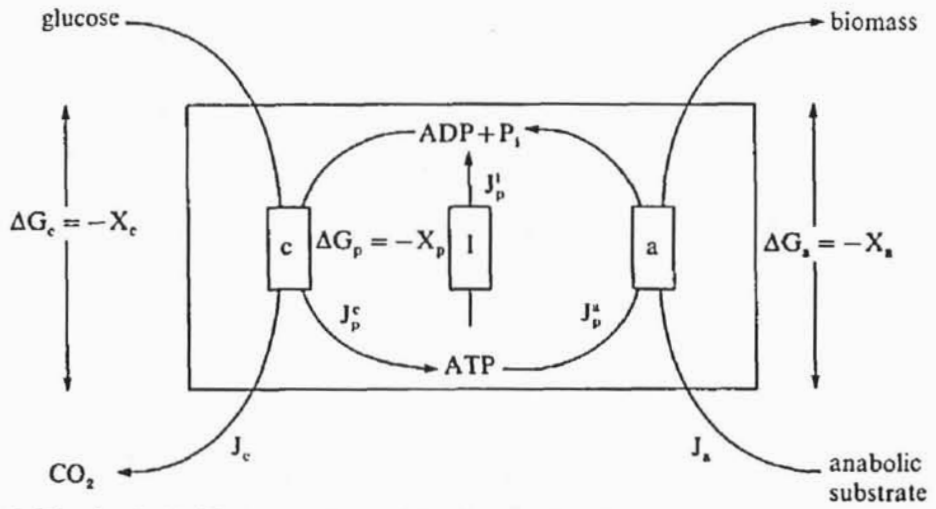


Fig. 7. The simple MNET description of microbial growth, illustrated for an aerobic heterotrophic cell. In this description, it is considered that catabolism (c) is coupled to anabolism (a) via the adenine nucleotide system (p). The leak (l), formally modelled as an uncoupled ATP hydrolase activity, indicates that the couplings are imperfect. The forces and cognate fluxes of the partial reactions are given by the affinities X and the fluxes J, whilst the overall thermodynamic efficiency of a linear energy converter of this type is equal to $-J_aX_a/J_cX_c$.

medium (Andersen & von Meyenburg, 1980; Harvey & Koch, 1980; and see Koch, 1985). More recently, we have shown (J. Harris, D. B. Kell & J. G. Morris, in preparation) using an inhibitor titration approach, that (as might be expected from the overview of the control analysis given above) for *Clostridium pasteurianum* growing in unrestricted batch culture in glucose minimal medium, *both* catabolism and anabolism exert a significant flux-control. Analysis of this point was made particularly straightforward in that the rate of ATP production could be assessed by following the rate of fermentation end-product formation in this obligately fermentative organism. Thus, the addition of an inhibitor acting selectively against catabolism or of one acting selectively against anabolism (Figs. 8(a, b)) leads to an immediate decrease in the rate of growth, and the fact that these inhibitors *are* selective for catabolism or anabolism, at least during the early stages of a titration, is borne out by their effects upon the calculated rate of ATP synthesis determined (from the rate of fermentation end-product formation) in parallel (Fig. 8(c)). It should be mentioned that the sum of the flux-control coefficients for catabolism and anabolism does not equal one (and will always exceed it) since there is an imperfect coupling between ATP synthesis and its utilisation in biomass formation. Indeed, stimulating the 'energy leak' by the addition of small concentrations of the protonophorous uncoupler tetrachloro-salicylanilide also leads to an

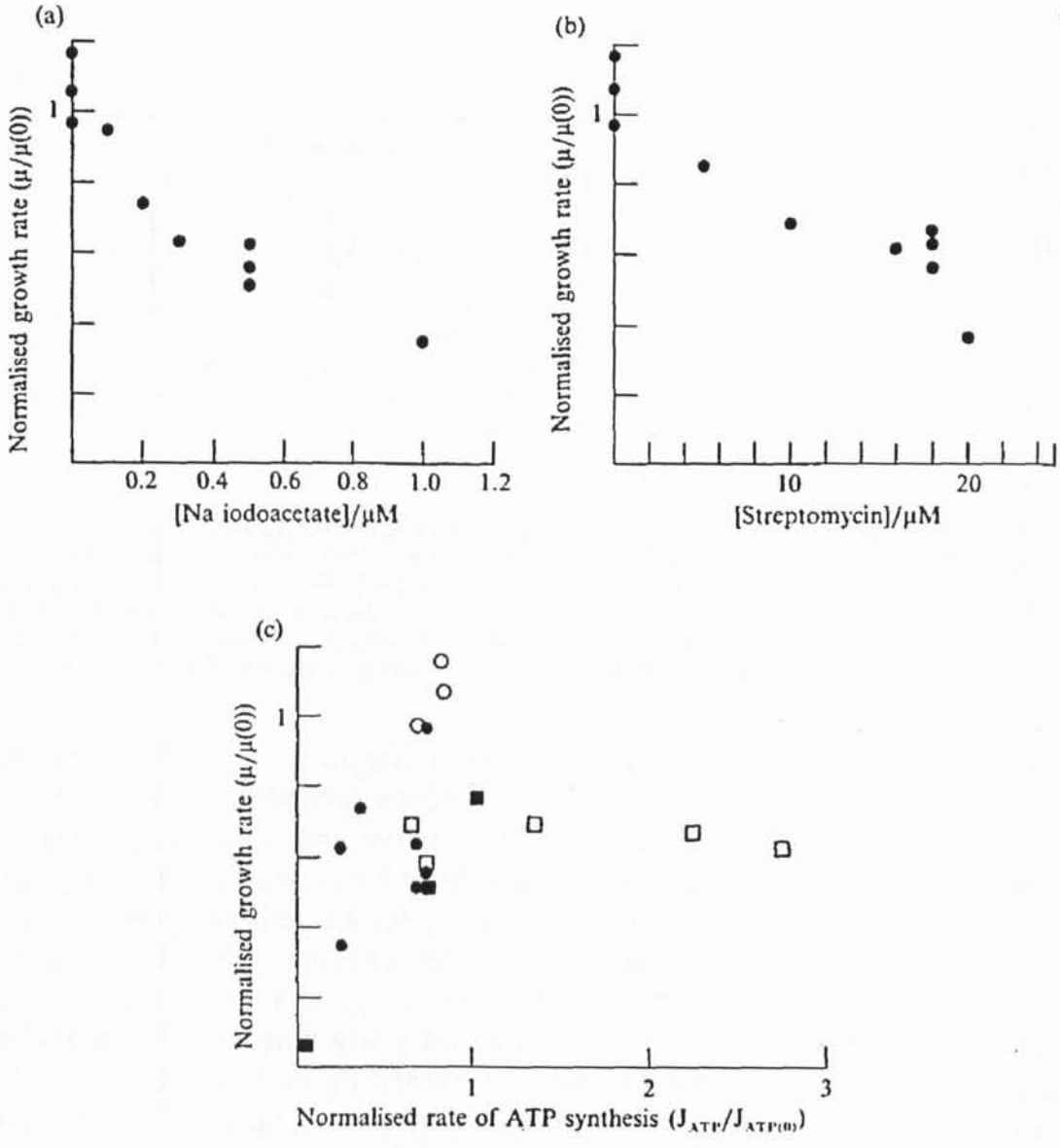


Fig. 8. Both catabolism and anabolism can contribute to the control of microbial growth rate in unrestricted batch culture. In the experiments illustrated (J. Harris, D. B. Kell & J. G. Morris, unpublished observations), *Clostridium pasteurianum* MR505 was grown anaerobically in glucose minimal medium (Clarke *et al.*, 1982). Growth was measured turbidimetrically and the catabolic rate gas chromatographically by the increase in fermentation end-products. When the optical density had reached 0.8, an appropriate titre of the catabolic inhibitor sodium iodoacetate or of the anabolic inhibitor streptomycin was added to the culture, and the change in growth and catabolic rates monitored for 30 minutes. J_{ATP} (in nmol (min mg dry weight)$^{-1}$) was determined from the known stoichiometries of glycolytic ATP formation (Morris, 1986). (a) immediate decrease in normalised growth rate upon inhibition of catabolism with iodoacetate; (b) Immediate decrease in normalised growth rate upon inhibition of anabolism with streptomycin; (c) relationship between normalised growth rate and normalised rate of ATP synthesis, to illustrate the fact that the latter is respectively decreased and increased by the catabolic and anabolic inhibitors. Data are from (a) (controls (○) and iodoacetate titrations (●)) and (b) (streptomycin titrations (□)), and from titrations with tetrachlorosalicylanilide (■). $\mu(0)$ and $J_{ATP(0)}$ represent the growth and ATP synthetic rates prior to the addition of inhibitor. Each point represents a separate culture.

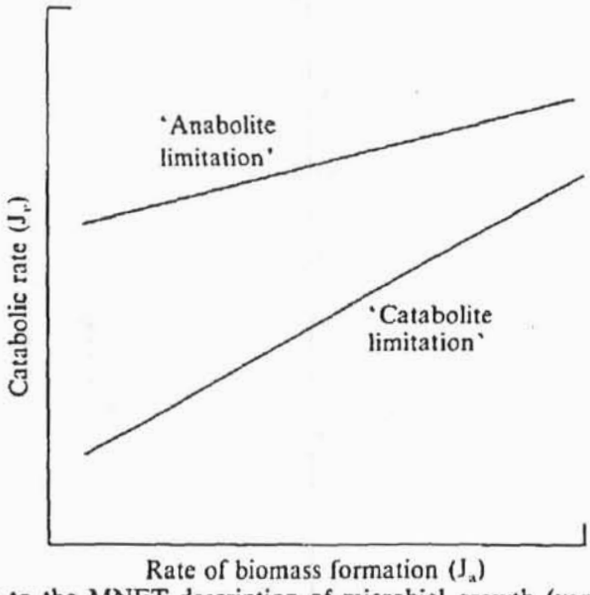


Fig. 9. According to the MNET description of microbial growth (van Dam *et al.*, 1988), the relationship between the catabolic rate (J_{ATP}) and the anabolic (growth) rate depends upon whether the cell is more anabolite-limited or catabolite-limited. The theoretical coupling stoichiometry in the absence of any 'leak' reactions (i.e. the slope of such a plot) lies between these boundary values, and the line passes through the origin.

immediate inhibition of the growth rate (data not shown), suggesting that the 'leak' also contributes (with a negative control coefficient) to the control of growth rate under the conditions stated.

Another example of the contribution of 'leak' to the overall metabolism of a bacterium can be found in the study of Mulder *et al.* (1986, 1988). The presence in *E. coli* of two K^+ uptake systems with a different affinity and – presumably – a different stoichiometry ($\rightarrow K^+$/'ATP equivalent') may lead under certain circumstances to a futile cycling of K^+ between the internal and external phases of the cell. The gradient of K^+ across the membrane determines the rate of the ATP-dissipating cycle, the rate being maximal when the gradient is high. Experimentally, this is seen by comparing the metabolism and energetics of the wild-type strain, having both uptake systems, with a mutant strain lacking one of the K^+-uptake systems. It is found (Mulder, 1986, 1988) that the wild-type strain has a higher rate of catabolism, and the difference in the rate of substrate utilisation increases with increasing growth rate (which is paralleled by an increasing gradient of K^+ across the cell membrane).

Treating metabolism according to the MNET formalism (Fig. 7), it may be deduced (van Dam *et al.*, 1988) that a plot of the rate of catabolism versus the rate of anabolism gives an indication as to what factor more limits growth (Fig. 9). Under 'anabolism-limited'

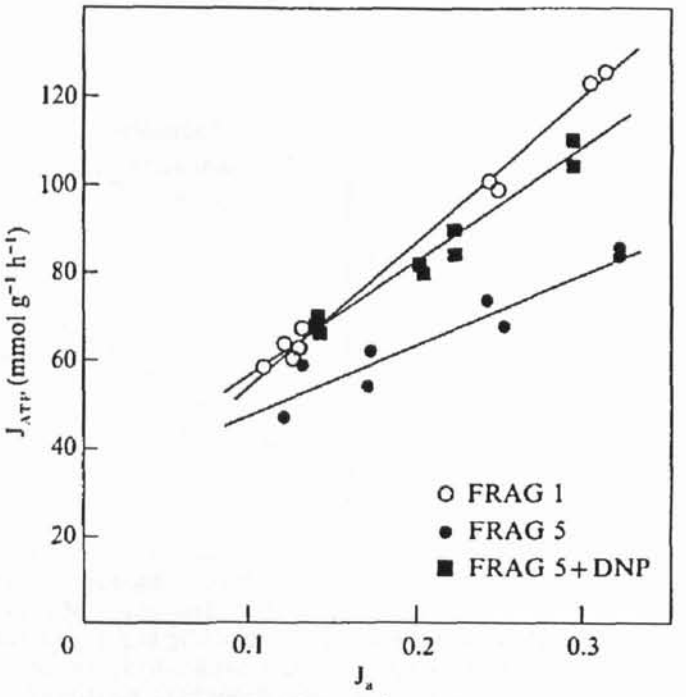


Fig. 10. Effect of removal of the high-affinity K^+ uptake system from an *Escherichia coli* strain on its metabolic behaviour in K^+-limited chemostat cultures. *E. coli* wild type (FRAG-1, open circles) and a mutant lacking the high-affinity K^+ uptake system (FRAG-5, closed circles) were cultured aerobically in K^+-limited chemostat cultures. It is clear that the wild-type requires more ATP-equivalents to produce the same amount of biomass per unit time. The presence of an appropriate titre of the uncoupling agent 2,4-dinitrophenol in FRAG-5 cultures (closed squares) leads to a behaviour similar to that of the wild-type strain, indicating that the wild-type strain is indeed metabolically uncoupled (undertaking 'futile' cycling) under these conditions. From Mulder (1988).

conditions, the slope of this relation is less than the 'theoretical stoichiometry', i.e. the coupling to be expected in the absence of any ATP-leak. Under 'catabolism-limited' conditions, however, the slope is larger than the theoretical stoichiometry. From the experimental point of view, an increase in the ATP-leak should increase the slope when catabolism is most limiting but decrease it if anabolism is most limiting. The experimental finding with *E. coli* growing in the chemostat with limiting amounts of K^+ is very clear (Mulder, 1988, p. 70) (Fig. 10): the introduction of futile cycling leads to a steeper slope, i.e. K^+ limitation corresponds more to a situation of catabolic limitation. Unfortunately it has not yet proved possible to regulate the activity of the futile K^+ cycle independently (by varying the amount of transporter) so as to determine a control coefficient for the leak.

The presence of these types of leaks may be very important for microorganisms; as discussed elsewhere (Westerhoff *et al.*, 1983),

microorganisms may adapt to environmental conditions by changing the coupling in the different elements of their metabolic network. Thus it appears that microorganisms have in general evolved to possess an optimal efficiency of growth yield at maximal growth rate (see Westerhoff *et al.*, 1983; Westerhoff & van Dam, 1987; Kell, 1987); evidently some yield has been sacrificed to increase the rate of production of new biomass.

In pathways with branches, i.e. in most real metabolic pathways, there is more than one flux. The distribution of the control amongst the enzymes tends to be different for the different fluxes (Westerhoff & Kell, 1987; Westerhoff & van Dam, 1987). Thus if one wishes to optimise a microorganism by increasing the activity of one of its enzymes, the enzyme to be selected will of course depend upon the 'purpose' of the microbial process.

For the biotechnologist, the above considerations are very important in the selection of microorganisms for different purposes. For instance, in processes for the removal of BOD in sewage it is very desirable to have a population of cells which metabolises rapidly yet has a poor biomass yield. The same is true for fermentations such as that used to produce ethanol, where the well-known differences in glycolysis (and their ATP yield) for *Zymomonas* versus *Saccharomyces* strongly favour the former organism so far as volumetric productivity is concerned (Rogers *et al.*, 1980, 1982; Morris, 1986). For this type of process one is interested in cells with large ATP-leaks. Conversely, for the production of single-cell protein it is advisable to have a small ATP-leak but one still sufficiently large to permit maximal growth rate. The classic study of Windass & colleagues (1980) provides a suitable illustration of the improvements in biomass yield to be expected when ATP-leaks are decreased, even under carbon-limited conditions.

Although, as mentioned above, no studies yet published have sought to account, within the framework of MCT, for improvements in the flux towards metabolites of commercial importance consequent upon the amplification of a stated gene, several examples in which the approximate approach was taken may be cited. Thus, Dauce-Le Reverend *et al.* (1982) give an early and related example for lysine, by cloning different enzymes of lysine biosynthesis onto a plasmid. More recently, Sano *et al.* (1987) amplified a cloned PEP carboxylase gene in *Brevibacterium lactofermentum* to improve the production of proline and of threonine; inspection of their data indicates that the flux-control coefficient of this enzyme in the former

case must have been nearly unity. Tsuchida & Momose (1986), from the same laboratory at Ajinomoto, succeeded in improving leucine production in a strain of the same organism to $34\,g\,l^{-1}$, which is claimed to be a record. Thus, and given the ability to combine pathways from different hosts (Anderson *et al.*, 1985; Stellwag & Brenchley, 1986), it is clear that almost unlimited possibilities for rational approaches to improving fermentation yields are now in prospect (Beppu, 1986; Kisumi, 1986; Stellwag & Brenchley, 1986; Byrom, 1987). Martin (this volume) and Niederberger (this volume) describe progress in the improvement of amino acid fermentations by such means. An appreciation of the MCT will permit a rational approach to the taking of decisions concerning which enzymes should be cloned and/or over-expressed (Kell & Westerhoff, 1986*a*, *b*; Westerhoff & Kell, 1987; Westerhoff & van Dam, 1987).

CRITIQUE AND LIMITATIONS OF MCT, AND ALTERNATIVE APPROACHES

The summation and connectivity properties are laws that are strictly valid in the sense that they can be proven mathematically. Indeed, the theorems of metabolic control theory are valid for linear and branched pathways alike (cf. Meiske & Reich, 1987). The price one has to pay for this is that MCT in its exact form is confined to metabolic systems that meet a number of stringent requirements. Moreover, MCT in its present form can answer only a subset of all the questions that one could conceivably ask with respect to metabolic control. In practice, the ensuing limitations of MCT are virtually insignificant by comparison with the uncertainties imposed by the lack of precise and complete experimental knowledge about the system of interest. In fact, quite a few of the perceived (Crabtree & Newsholme, 1987) limitations can be avoided by defining the system in a slightly different manner, as discussed above for cases in which the concentration of pathway substrate is not constant or where the system is apparently not steady. In cases where the activity of two enzymes is genetically linked (i.e. they are pleiotropically induced), the percentage effect of the inducer may still be evaluated using MCT, simply by summing the two effects. Similarly, if an inhibitor is not entirely specific for a target enzyme its effect on a flux may be predicted by multiplying the elasticity coefficient of each affected enzyme by the flux-control coefficient of the enzyme and summing these. The limitation that elasticity coefficients are defined at constant concentrations of all

other intermediates (and effectors) may be removed by allowing some of the latter to relax to a new steady state (Kacser, 1983; Westerhoff, 1983; cf. Westerhoff & van Dam, 1987).

A more substantial limitation of the MCT is that the answers it gives do not say everything one would wish to know about the control of metabolism, for instance in long-term changes due to induction or repression. A second set of limitations is that MCT does not address the *absolute magnitudes* of fluxes and metabolite concentrations, since it only considers small changes in them. In linear pathways, the predictions of MCT would be correct for larger changes if the concentration-dependencies of the reaction rates were to conform to power laws. Savageau (1972, 1976; Savageau & Voit, 1982) has developed a Biochemical Systems Theory (BST) based on the approximation of unidirectional reaction rates by power laws (see also Crabtree & Newsholme, 1985), such that in the limit of very small changes the conclusions of this theory are as exact as, and analogous (though not identical) to, those of MCT. The parameters used in BST are not quite the same as those used in MCT, which are more directly amenable to manipulation.

Mosaic Non-equilibrium Thermodynamics (MNET) is another formalism designed to describe the absolute magnitude of fluxes (Hellingwerf *et al.*, 1982; Westerhoff *et al.*, 1982; Westerhoff & van Dam, 1987; van Dam *et al.*, 1988). This method, in which the severely limited proportional and symmetrical flow–force relationships of the Onsager type of non-equilibrium thermodynamics (Caplan & Essig, 1983) have been replaced by more-generally valid piece-wise-linear relations between reaction rates and the free energies of reaction, is also exact for small changes but becomes approximate for larger ones. MNET, in which parameters such as the activities of enzymes and the free energy of ATP hydrolysis are explicit, can account for why the observed efficiencies of microbial growth fall short of their theoretical maxima (see above).

Stressing that concentrations of pathway substrates may not be constant, and that changes in their concentrations are not likely to be small, Newsholme, Crabtree and colleagues (Crabtree & Newsholme, 1985, 1987) prefer to model so-called flux-generating parts of metabolic pathways. This work has contributed greatly to the understanding of the possible regulatory roles of apparently futile cycles. Although an opposite impression may have been generated (Crabtree & Newsholme, 1987; Kacser & Porteous, 1987), this work and MCT may be regarded as complementary to each other, once

it is recognised that the former concentrates on parts of metabolic pathways that have high flux-control coefficients and can be treated as if in isolation, an approach also fostered by Kacser (1983, 1988) and Westerhoff *et al.* (1987*a*, *b*).

There are a number of limitations that MCT shares with all other approaches. One of these is that it considers a population of cells to be homogeneous (i.e. an ensemble). Clearly in a growing bacterial culture there may be substantial metabolic differences between the individual cells in terms of their response to identical stimuli, for instance because of differences in their phase of the cell cycle (e.g. Mitchison, 1971; Donachie *et al.*, 1973; Lloyd *et al.*, 1982); our knowledge of the significance of this in biotechnological processes (Koplove & Cooney, 1979) remains woefully poor. Indeed, Skarstad *et al.* (1983) found that even during strictly exponential growth of batch cultures of *E. coli* over four decades of concentration (as determined turbidimetrically) the culture was highly inconstant with respect to the distribution of cellular DNA content. As is well known, the expression of particular enzymes in microbial cultures is an extremely strong function of the exact physiological state of individual cells, even when the enzyme of interest is cloned onto a high-expression plasmid vector (Seo & Bailey, 1986), whose loss is itself highly physiological-state-dependent (Caulcott *et al.*, 1987). In fact, the further development of MCT seems necessary here, since the theory as it stands does not treat enzyme concentrations as variables.

MAXIMUM THEORETICAL RATES OF BIOTRANSFORMATIONS

Given the fact that MCT does not of itself consider the *absolute* fluxes, even though changes (improvements) are what in general matter most in biotechnology, it is worth drawing attention to some of the relevant considerations. (For reasons of space, we do not here consider the optimal *structure* of metabolic pathways: Waley, 1964; Heinrich & Holzhütter, 1985). Even if a fermentation produces an end product at a concentration of $80\ g\ l^{-1}$, the volumetric productivity may approximate only some $1\ \mu mol\ (litre\ culture.s)^{-1}$, if this takes 10 days to accomplish (Suckling, 1984), corresponding to a flux probably not exceeding $10\ nmol\ min^{-1}\ mg^{-1}$ protein (Kell & Westerhoff, 1986*b*). The maximum fluxes of primary metabolic pathways, such as glycolysis in *Zymomonas* (Rogers *et al.*, 1980, 1982), are only some $3\ \mu mol\ min^{-1}\ mg^{-1}$ protein, so that if glycolytic enzymes *in toto* constitute 10% of the cell protein and have an average M_r

of say 20 kD, the average turnover number is some $100\,s^{-1}$. The theoretical maximum rate for enzymatic turnover depends upon the chemically achievable values of k_{cat}/K_m and the local viscosity (which latter affects the rate of substrate diffusion), but for *the very few* evolutionarily optimised enzymes exceeds $10^6 s^{-1}$ (Albery & Knowles, 1976; Fersht, 1985). Notwithstanding the facts that *in vivo* evolutionary selection works on pathways, not enzymes (Welch & Keleti, 1981), and that increased fluxes are necessarily associated with increased cooling requirements, it is clear that we have a long way to go before we may state that our fermentations have attained their maximal, theoretical volumetric productivities. The Metabolic Control Theory and other rational approaches may assist us in getting there.

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