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Strategies for Manipulating Metabolic Fluxes in Biotechnology[1]

ATHEL CORNISH-BOWDEN,* JAN-HENDRIK S. HOFMEYR,† AND MARÍA LUZ CÁRDENAS*

*Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, 31 chemin Joseph-Aiguier, B.P. 71, 13402 Marseille Cedex 20, France; and †Department of Biochemistry, University of Stellenbosch, Stellenbosch, 7600, South Africa

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Strategies for biotechnologically manipulating metabolic fluxes are critically examined in relation to a model system. The common idea of first identifying the rate-limiting enzyme in the biosynthetic pathway to a desired end-product, and then increasing its activity, is shown to be completely ineffective; such manipulation typically produces only trivial changes in flux. Manipulating the activities of all of the enzymes in a biosynthetic pathway by amounts calculated to increase a desired flux while leaving all other fluxes and all concentrations unchanged is potentially effective, and can be applied to any system without regard to its regulatory design. however, it requires accurate knowledge of the initial state of the system and the ability to make precise changes to numerous activities. The classical information about the regulatory mechanisms that exist in living organisms suggests that one can make much simpler manipulations, involving only the steps that remove the desired end-product, with almost equally satisfactory results. © 1995 Academic Press, Inc.

INTRODUCTION

The possibility of using genetic manipulation to increase selected enzyme activities, essentially at will, appears at first sight to have opened the door to the application of classical ideas of enzyme regulation to useful industrial objectives. If one can identify the enzyme that catalyzes the rate-limiting step in the normal biosynthetic pathway to a valuable end-product, it would seem straightforward to clone its gene and use the standard techniques of genetic manipulation to produce a greatly increased activity in the organism, with much higher yields of the desired metabolite. In this article we use examples from the literature to show that this approach does not work in practice, we discuss in terms of metabolic control analysis why it cannot be expected to work in the future, and we consider alternative approaches that may have better prospects for success.

A convenient experimental example is provided by a recent study of tryptophan biosynthesis in yeast, for which the biosynthetic part of the pathway (Fig. 1a) consists of five enzymes. In an initial set of experiments the activities of four of these were increased individually by factors of 10 to 50, and in other experiments all of them were similarly increased in various combinations (*1*). These experiments have been

[1] This paper is dedicated to Professor Jeremy Knowles on the occasion of his 60th birthday.

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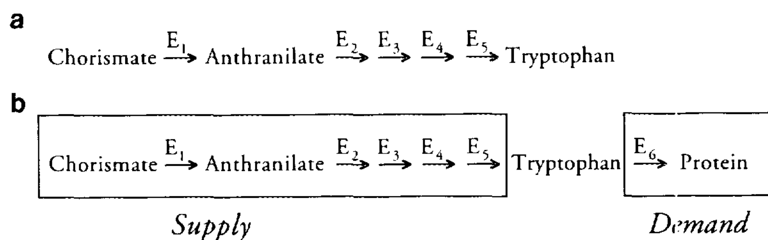

FIG. 1. Biosynthesis of tryptophan in *Saccharomyces cerevisiae*. The identities of the enzymes E_1 to E_5 are as follows: E_1, anthranilate synthase; E_2, anthranilate phosphoribosyl transferase; E_3, phosphoribosylanthranilate isomerase; E_4, indoleglycerol phosphate synthase; E_5, tryptophan synthase. E_6 represents not only tryptophan tRNA synthetase but also all of the subsequent process of protein synthesis. (a) Biosynthetic pathways are usually drawn in textbooks without explicit indication of the reactions that consume the "end-product." (b) However, regulation of such a pathway involves communication via the end-product between a demand block and a supply block. In this example only the most important use of tryptophan is shown as the demand block; other pathways, such as degradation to tryptophanol, constitute competing demand blocks.

discussed elsewhere from the same point of view as the present article (*2*), and only a summary need be given here: increasing any single enzyme activity, by factors in the range of 10- to 50-fold, had no significant effect on the flux to tryptophan; however, when all five activities were increased by factors of about 20 (or all five apart from phosphoribosylanthranilate isomerase, which appears to have little influence on the flux) there was a substantial increase in flux, albeit considerably less than the increase in the activities of the pathway enzymes. This study is the most detailed that can be found in the literature, but others have led to equally disappointing results. For example, similar efforts to improve the yield of ethanol in alcoholic fermentation of yeast gave results essentially the same as those for tryptophan biosynthesis (*3*).

Metabolic control analysis originated in articles written more than 20 years ago (*4, 5*), but it remains relatively unknown to many biochemists and nearly all biotechnologists. Yet it readily explains the disappointing results of attempts to improve yields by increasing activities of rate-limiting enzymes. The essential point is that the classical conception of a rate-limiting enzyme that completely determines the flux through a pathway is an oversimplification; instead, the control of flux in any pathway is shared among all the enzymes of the organism, in proportions that cannot be predicted *a priori*. Moreover, even if one enzyme happens to have a large share of the total flux control, this share always decreases if its activity is increased. Clear experimental examples of this sort of behavior can be found in studies of arginine biosynthesis in *Neurospora crassa* (*6*).

Westerhoff and Kell (*7*) have described how metabolic control analysis can be applied to biotechnological questions, but it has yet to have a major impact on the practice of biotechnology; efforts to use genetic engineering to realize commercial objectives have been guided more by a vague optimism than by application of the principles of metabolic control.

Metabolic control analysis offers a powerful means of examining the general properties of metabolic systems, but its very generality means that it may overlook some of the specific properties of real systems. In particular, it makes no assumptions about the existence of the regulatory properties of real enzymes, and thus does not take advantage of the knowledge of the regulatory design of biological systems that has accumulated during the past 40 years—feedback inhibition, allosteric and cooperative interactions, etc. (*8–11*). Consequently, although techniques based on control analysis for altering fluxes and metabolite concentrations may well be effective they are likely to be more complicated, and hence experimentally more difficult, than necessary. In this paper we examine a model system to illustrate this point.

In a previous work (*12*) we argued that the classical mechanisms are essential for achieving satisfactory regulation, but to be properly understood they need to be considered in the framework of a systemic theory of control such as metabolic control analysis. Most pathways can be considered to consist of a *supply block*, consisting of a series of reactions leading to a particular end-product, and a *demand block*, consisting of the reactions that consume the end-product. (In reality an "end-product" is not the end of metabolism but the link metabolite between the two blocks. The term is thus misleading, but as it is universally used in this sense in biochemistry we shall retain it here.)

The demand block is commonly omitted from textbook illustrations of metabolic pathways (which are thus shown as in Fig. 1a rather than as in Fig. 1b). Inclusion of the demand block is necessary, however, for understanding metabolic regulation, because the usual effect of feedback inhibition of the first committed step in a pathway by its end-product is to transfer much of the control into the demand block from the enzymes of the supply block. In this way they allow precursors for other pathways to be provided as they are required. This point was made in one of the original articles on metabolic control analysis (*4*), but it was given little emphasis and has largely been ignored by later workers. In a recent article Kacser (*13*) even describes the concept of a regulatory enzyme as one of the "victims" of metabolic control analysis, implying that it is a concept that could usefully be discarded.

Nonetheless, simulations of various metabolic systems have shown that the classical regulation mechanisms do have an important role in metabolic regulation (*2, 12, 14*). This role is essentially as it was proposed 20 years ago (*4*), i.e., the transfer of control from supply to demand, but there has been an unexpected result, namely that cooperativity and feedback inhibition are far more important for the concentration of the feedback inhibitor than for the flux through the pathway. In other words fluxes can be regulated reasonably effectively without either cooperativity or feedback inhibition, but only at the expense of very large changes in metabolite concentrations.

METABOLIC CONTROL ANALYSIS

The terminology and principal ideas of metabolic control analysis are thoroughly discussed in various recent reviews (*15, 16*), and only a very brief summary will be

given here. The initial objective was to move away from the traditional mechanism-based view of metabolism toward a *systemic* view, in which kinetic behavior is discussed in terms of how the whole system responds to a change in the parameters that define its environment. If a change in some parameter p changes the rate v_i of the ith enzyme (E_i) when considered in isolation from the pathway (i.e., in the presence of all metabolites that influence it, at the concentrations that occur in the complete system, but in the absence of all other enzymes), then the sensitivity of a flux J through the system can be expressed in terms of a *flux control coefficient* (*17*):

$$C_i^J = \frac{\partial \ln J}{\partial p} \bigg/ \frac{\partial \ln v_i}{\partial p}. \qquad [1]$$

The identity of the parameter p does not have to be specified, because the value of C_i^J does not depend on it. If, as is often but not always the case, the rate v_i is directly proportional to the total concentration e_i of E_i, then it may be convenient to take p as identical to e_i, in which case C_i^J may be defined more simply. However, although this type of definition was used originally (*4*), it led to a widespread misunderstanding that metabolic control analysis deals only with effects brought about by changes in enzyme concentrations or changes in limiting rates of enzyme reactions. The present trend in metabolic control analysis is to use the more general definition given in Eq. [1].

Control coefficients are defined similarly for other kinds of variables; for example, a *concentration control coefficient* refers to effects on the concentration s_j of an intermediate S_j:

$$C_i^{s_j} = \frac{\partial \ln s_j}{\partial p} \bigg/ \frac{\partial \ln v_i}{\partial p}. \qquad [2]$$

In the standard mechanistic approach to enzymology the kinetic properties of enzymes are expressed in terms of Michaelis constants, inhibition constants, limiting rates, etc. For purposes of metabolic control analysis, however, it is more convenient to decrease the emphasis on mechanism by using quantities that relate effects to causes in a more phenomenological way, similar in definition to control coefficients:

$$\varepsilon_{s_j}^{v_i} = \frac{\partial \ln v_i}{\partial \ln s_j}. \qquad [3]$$

This quantity $\varepsilon_{s_j}^{v_i}$ is called the *elasticity* of v_i with respect to s_j. As this term can seem rather obscure it is helpful to realize that it is the same as the more familiar concept of *order of reaction*, which for enzymes is never a constant, as it depends on the concentrations of substrates, products, and other effectors.

It is obvious that the properties of a system must depend on the properties of its components, but the form of the dependence is less obvious. The important relationships for an introductory understanding of control analysis are the *summation relationships*, which are as follows,

$$\sum_{\text{all steps}} C_i^J = 1, \qquad [4]$$

for any flux J, and

$$\sum_{\text{all steps}} C_i^{s_j} = 0, \qquad [5]$$

for any metabolite concentration s_j. In a simple system, summation over all steps is the same as summation over all enzymes, but in more complex cases it also includes transport steps and allows for the possibility that one enzyme may catalyze more than one step. Provided that one accepts that a control coefficient does in some way measure control, i.e., that it is not just a name, Eq. [4] immediately establishes the crucial fact about flux control, that it is shared among all the enzymes in a system, and in a system of n enzymes the mean flux control coefficient is $1/n$, though as control is not shared evenly individual values can be far from the mean. The existence of negative flux control coefficients in branched pathways implies that the mean of the absolute values may be substantially larger than $1/n$. Nonetheless, in experimental and model systems that have been studied it is rare to find enzymes with flux control coefficients that approach or exceed unity.

It is important to realize that the properties of a system expressed by Eqs. [4] and [5] and similar relationships are independent of any specific regulatory properties of the component enzymes, and although such properties may well exist and affect the distribution of control among the enzymes, striking regulatory properties do not guarantee high flux control coefficients for the enzymes that possess them. For example, despite the well-known sensitivity of phosphofructokinase to various effectors, it is quite misleading to regard it as the primary controlling enzyme in glycolysis: in studies of glycolysis in the human erythrocyte, under various conditions of pH, temperature, and phosphate concentration, it always had flux control coefficients in the range 0.1–0.3, compared with values in the range 0.7–0.9 for hexokinase (*18*).

MODEL

We shall examine the possible ways of manipulating metabolic fluxes on an industrial scale in relation to the model pathway shown in Fig. 2, which is more complicated (and hence, we hope, closer to reality) than those studied in previous works (*2, 12, 14*). It shows the biosynthesis of two end-products, S_{4a} and S_{4b}, from a precursor, X_0, via a branch-point metabolite, S_2. A different type of symbol is used for the precursor to emphasize that it is considered external to the system, i.e., its concentration is fixed and does not depend on the properties of the eight enzymes in the system, whereas all of the other metabolite concentrations are set by the system itself. Although inevitably the numerical values assumed for the eight kinetic equations are arbitrary, the essential features of the model are not arbitrary, and correspond to a typical case of sequential feedback regulation, as discussed in Stadtman's classic article on metabolic regulation (*19*): each of the two end-products inhibits the first committed step of its formation, and the branch-point metabolite inhibits the first step of the whole pathway. Other regulatory designs are, of course, possible (*19*), but similar types of behavior result, in that the normal tendency of

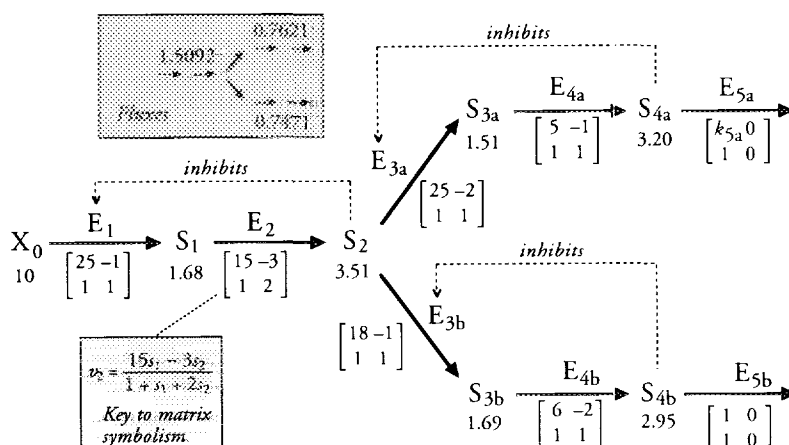

FIG. 2. Model of a branched metabolic pathway. There are two end-products, S_{4a} and S_{4b}, and two demand blocks, represented by the enzymes E_{5a} and E_{5b}. Each end-product inhibits the first committed step of its synthesis, and in addition the branch-point metabolite S_2 inhibits the first reaction of the pathway. Each reaction obeys the reversible Michaelis–Menten equation, $v = (c_1 s + c_2 p)/(1 + c_3 s + c_4 p)$, where s and p are the concentrations of the immediate substrate and product of the reaction, respectively, and the coefficients c_1 to c_4 are shown as matrices under the reaction arrows. (Note that c_2 is always negative or zero, i.e., the second term of the numerator of the rate expression is never positive). In the case of E_2 the correspondence between matrix and equation is shown explicitly in a shaded box below. For each of the reactions subject to feedback inhibition the denominator of the rate equation is modified by the inclusion of an extra term, x^4, where x is the concentration of the inhibiting molecule. The pool concentration (x_0) was held constant at a value of 10, and the steady-state concentrations of the intermediate metabolites, shown under each metabolite, were calculated with the program MetaModel (*20*), for a value of 1 for k_{5a}. The steady-state fluxes were as shown in the upper shaded box, i.e., 1.5092 for the common flux, and 0.7621 and 0.7471 for the fluxes through branches a and b, respectively. This steady state was taken as a starting point for examining the effects of various modifications.

the feedbacks is to transfer control to the demand steps, shown in Fig. 2 in vestigial form as the irreversible reactions catalyzed by E_{5a} and E_{5b}.

METHOD

All simulations were done on an IBM PC-compatible computer using the metabolic simulation program MetaModel (*20*).

RESULTS

Figure 3 illustrates the importance of the feedback loops for satisfactory regulation. When they are present (Figs. 3a,3b), a 5-fold increase in the demand for S_{4a} results in a 4.1-fold increase in the flux through branch a, but less than a 2% decrease

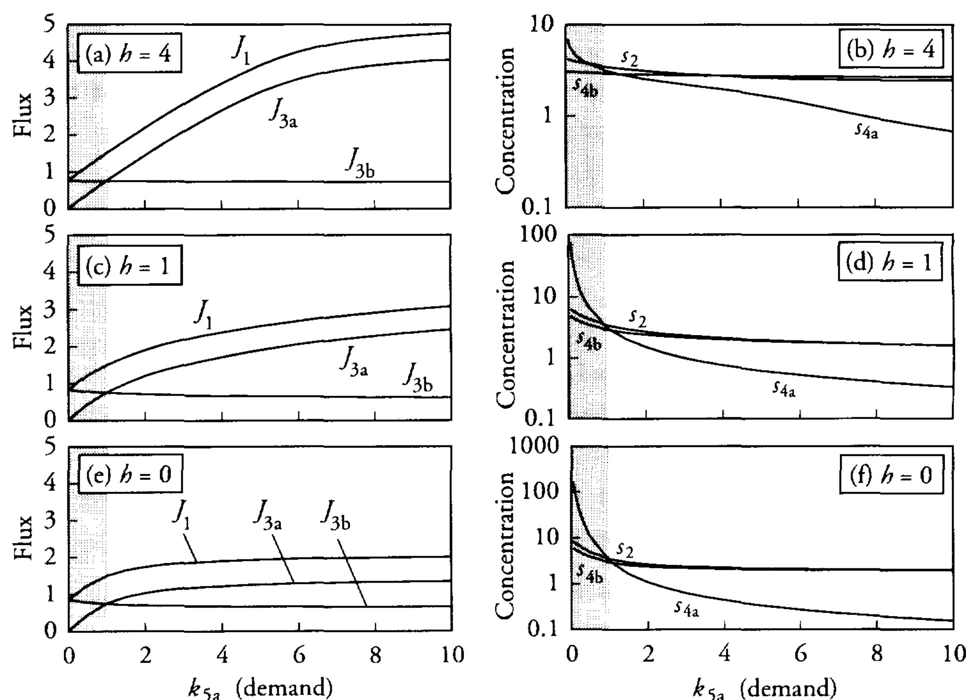

FIG. 3. Effect of feedback inhibition and cooperativity on metabolic fluxes and concentrations. The factor k_{5a}, a measure of the demand for S_{4a}, was increased by factors up to 10 (unshaded regions), or decreased by factors up to 20 (shaded regions), from the initial value of 1, and the effects on (a) fluxes and (b) certain metabolite concentrations are shown. In (c) and (d) the same variations in k_{5a} were examined, but the model was altered so that the feedback inhibitor concentration appeared in each relevant equation raised to the power 1 instead of 4, and the coefficients of these concentrations were increased by the factors needed to give exactly the same steady-state fluxes and concentrations in the starting state with $k_{5a} = 1$. In (e) and (f) the same variations in k_{5a} were examined, but the model was altered so that the feedback inhibitions were eliminated entirely and the denominator coefficients of the immediate products of the three reactions were increased sufficiently to give exactly the same steady-state fluxes and concentrations in the starting state with $k_{5a} = 1$.

in the flux through branch *b*, with decreases in the intermediate concentrations of 47% for S_{4a}, 24% for S_2, and 7% for S_{4b}. If the feedback effects are made noncooperative (Figs. 3c,3d) the behavior is less satisfactory: the increase in the flux through branch *a* is only 1.7-fold, and the effects on metabolite concentrations are much larger, with that of S_{4a} decreasing 5-fold. This trend continues if the feedback effects are eliminated entirely (Figs. 3e,3f): only a 30% increase in flux is then achieved by increasing demand 5-fold, and this is accompanied by a more than 9-fold decrease in the concentration of S_{4a}. As all of the lines in Fig. 3 are curved, effects at other increases in demand are not simply proportional to the results for a 5-fold increase, but qualitatively all of the same trends continue.

The shaded parts of Fig. 3 show the effects of decreasing demand below the

starting value. Although this is less likely to be of interest in a biotechnological application, it is very important for the normal health of the organism, which must be able to switch off production of an end-product without enormously increasing its concentration. The lowest points plotted in the figure correspond to a demand of 5% of the starting value, and it may be seen (Fig. 3b) that the full system tolerates such a low level of demand very well, with little more than a 2-fold increase in the concentration of S_{4a}, whereas the less well-regulated systems do so very badly, with 24- and 53-fold increases in the same concentration with noncooperative feedback or no feedback, respectively (Figs. 3d,3f). Nonetheless, the three panels in the left half of the figure are very similar to one another, illustrating the point made earlier that there is a large range of demand levels in which the flux can be varied smoothly without any need for the classic regulatory mechanisms.

Various strategies have been used or suggested for producing large increases in selected metabolic fluxes. These will be referred to here as *opposition, oblivion, evasion, suppression,* and *subversion,* as in a previous discussion (*2*), the names being intended to suggest the implied attitude toward the regulatory mechanisms that operate in the native organism. Opposition is the only approach that has been widely attempted in practice: it consists of trying to force more metabolites through a pathway by increasing the activity of the enzyme in the supply block that is considered to be rate limiting, often assumed to be the enzyme subject to feedback regulation, i.e., E_1 or E_{3a} in the model of Fig. 2. Oblivion consists of constructing a control matrix and manipulating it mathematically to decide what changes in enzyme activity will produce the desired effects (*7*); although the regulatory mechanisms are taken into account by the matrix used in this approach, they are not easily visible to the human observer and are thus difficult to make use of in any explicit way. Evasion uses the fluxes in the starting state to calculate the changes in enzyme activity needed to produce the desired increase in flux while leaving all metabolite concentrations exactly unchanged, thereby avoiding triggering any regulatory effects that might oppose the desired manipulation (*1, 21*). Suppression eliminates the feedback loops that prevent brute-force opposition from working. Finally, subversion recognizes that the primary effect of feedback loops is to transfer control from supply to demand and accordingly seeks to increase fluxes by increasing the demand.

Application of the oblivion strategy, which has been described only in outline in the literature (*7*), appears to require complete analysis of the control structure of the system followed by manipulation of all of the component enzymes. We have not attempted it for the model of Fig. 2, but Table 1 shows the results of applying each of the other strategies. Suppression is an all-or-none strategy, i.e., any particular feedback loop is either present or absent. In the other cases the manipulations used were those intended to produce a fivefold increase in flux through branch *a*. As expected from the experimental cases of tryptophan and ethanol biosynthesis mentioned in the Introduction, opposition is largely ineffective: whether the activities of E_1, E_{3a}, or both are altered, the flux increases by only a few percent, and the small changes in flux that do occur are accompanied by changes of up to 66% in intermediate concentrations.

Evasion, by contrast, works exactly as expected, producing a fivefold increase in

TABLE 1
Results from Various Strategies for Increasing Flux through Branch *a* of the Model in Fig. 2

	Relative activity of each enzyme[a]									Relative metabolite concentrations			
Strategy	E_1	E_2	E_{3a}	E_{4a}	E_{5a}	E_{3b}	E_{4b}	E_{5b}	Relative J_{5a}	S_1	S_2	S_{3a}	S_{4a}
Wild-type	1	1	1	1	1	1	1	1	1.00	1.00	1.00	1.00	1.00
Opposition[b]	5	1	1	1	1	1	1	1	1.02	1.50	1.51	1.11	1.11
Opposition[b]	1	1	5	1	1	1	1	1	1.08	1.02	0.99	1.49	1.47
Opposition[b]	5	1	5	1	1	1	1	1	1.10	1.52	1.49	1.66	1.63
Evasion	3.02	3.02	5	5	5	1	1	1	5.00	1.00	1.00	1.00	1.00
Suppression	*	1	1	1	1	1	1	1	1.17	49.5	47.7	2.64	2.56
Suppression	1	1	*	1	1	1	1	1	1.29	1.06	0.96	17.8	16.9
Suppression	*	1	*	1	1	1	1	1	1.31	47.4	42.7	795	780
Subversion	1	1	1	1	5	1	1	1	4.13	1.74	0.76	3.66	0.53

[a] In cases marked * the activities of the enzymes were unchanged apart from omitting the feedback inhibition terms from the denominators of the rate equations.

[b] The three cases of opposition were also examined with much larger changes in activity, 100- instead of 5-fold in each case. Effects on the flux were only trivially different from those shown, but the effects on concentrations were much larger.

flux and no changes in metabolite concentration. However, it requires five different enzyme activities to be altered, by amounts that must be calculated exactly. The factors of 5 and 1 for the enzymes of branches *a* and *b*, respectively, are simply the desired increases in flux. In the common part of the pathway, the factor 3.02 is calculated from the fluxes in the starting state, as $(5J_{3a} + J_{3b})/(J_{3a} + J_{3b}) = (5 \times 0.7621 + 0.7471)/(0.7621 + 0.7471) = 3.02$. This calculation is easy to do for a fully defined model, but is likely to be more complicated in a real system, because the initial fluxes may not be accurately known, and there may be additional metabolic reactions that are not taken into account. Moreover, the more branches there are in the pathway from the initial reservoir to the desired end-product the greater the number of different factors that need to be calculated.

Suppression had only modest effects on flux in the example considered. Elimination of the feedback loop to E_{3a} gave a 30% increase in flux, accompanied by changes of around 17-fold in the concentrations of the metabolites in the branch affected. In a real system such large changes in concentration could be expected to produce undesired side effects, so that the actual flux increase might be less than calculated, or the viability of the organism could be compromised. Elimination of the feedback to E_1 gave only a 17% increase in flux with concentration changes of the order of 50-fold in the common part of the pathway. Eliminating both feedbacks produced scarcely any more effect on flux than eliminating that on E_{3a} by itself, and in this case the effects on the concentrations in the branch to S_{4a} were enormous, of the order of 800-fold.

Subversion seems to us to give the most satisfactory results. Although the effect on the desired flux is somewhat smaller than that for evasion, it is achieved by manipulation of only one enzyme activity instead of five, and the amount of manipulation does not need to be calculated exactly. It does produce appreciable changes in concentration, but these are not so large as to make unwelcome side effects a

near-certainty; they certainly appear negligible by comparison with the huge effects produced by suppression.

DISCUSSION

The results indicate that evasion and subversion are both effective strategies for increasing metabolic fluxes without undesirably large changes in metabolite concentrations. Evasion has the advantage that it makes no assumptions about the regulatory design of the metabolic system and is thus in principle applicable to any conceivable system. It does, however, require accurate knowledge of the reactions involved in the biosynthesis of the desired product, accurate knowledge of all the relevant fluxes, and accurate methods for altering all of the necessary activities by the calculated amounts. All of this may be quite difficult to achieve in practice, especially as the number of steps necessary to be considered may be much more than the five considered in the model.

Subversion, on the other hand, does involve assuming that the pathway of interest obeys the regulatory design features that have been found in real organisms, so it may give less satisfactory results than those of our simulation if the organism of interest is constructed in a different way. This, however, seems to us to be unlikely. Moreover, as subversion requires only one activity to be manipulated, by an amount that does not need to be calculated exactly, it may offer a much more practical approach to biotechnological problems than evasion.

In a real system one of the simplest ways of subverting a regulated system might be to engineer a permease mutant, so that the required product leaked into the medium; this would have important advantages apart from technical feasibility. As the cells would not need to be broken they could be maintained alive for longer productive periods, and isolation of the desired product from the medium would be easier than from a cell extract. Even the decrease in end-product concentration that accompanies subversion may be beneficial, as it could take advantage of the likely existence of mechanisms whereby gene expression is regulated by end-product; increased synthesis of biosynthetic enzymes would then help to overcome one limitation of subversion apparent in Fig. 3a, that of limited biosynthetic capacity.

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