A strategy for increasing an *in vivo* flux by genetic manipulations

The tryptophan system of yeast

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Decreases in enzyme activity often have little effect on the flux carried by the pathway. Similarly, up-modulation of single genes, and hence of the dependent enzyme concentrations, is frequently found to be ineffective in increasing the flux in the pathway in which the enzyme occurs. This insensitivity to enzyme variation is demonstrated experimentally for five separate enzymes in the tryptophan synthesis system of yeast, first by down-modulation of the gene dose and secondly by increasing the dose using multi-copy vectors. Such a lack of response is discussed in terms of the concepts of metabolic control analysis. When these five enzymes, however, were simultaneously increased by a multi-copy vector carrying all five genes, a substantial elevation of the flux to tryptophan was observed. These findings revealed a new phenomenon, namely the more than additive effects on the flux of simultaneous elevations of several enzyme activities.

INTRODUCTION

Recombinant DNA technology has opened up the possibility of altering phenotypes in specific and desired directions. Considerable interest resides in the increased production of primary and secondary metabolites, most usually in micro-organisms. Genes specifying enzyme activities in metabolic systems are therefore often regarded as potential targets for manipulation. Similarly, there are a number of medical and botanical applications which are considered to benefit from the increase in some internal metabolite or its rate of production.

The methodology of DNA manipulation is thought to be a short cut to the desired end. Additional copies of specific genes, so it is said, will result in direct improvement without the expensive trial-and-error or selection procedures previously undertaken. The first requirement, now almost routinely achievable, would therefore be the ability to identify, clone and insert additional copies of some particular gene which will result in increased enzyme activity at a particular step in the metabolic map. The second requirement is to know which gene or genes to choose. This is a problem outside the techniques and theories of molecular biology. It involves biochemical and physiological questions of whole systems, not simply knowledge of the individual gene–enzyme relationships. The present study addresses these questions and provides some relevant experimental evidence.

In work of this kind one can operate at different levels. When as geneticists we are considering an allelic substitution (whether in the nature or in the dose of a gene specifying an enzyme), then as biochemists we are considering a change in the rate of catalysis of the relevant step. As physiologists, however, we must consider the consequences of such a change in catalysis on the highly interactive remainder of the system.

Metabolism is a complex array of interlocked pathways with branch points, feedbacks, couplings, and so on. In principle, an event at a particular point reverberates throughout the whole system. If such an event is a change in enzyme activity, such a change would tend to change the concentrations of substrate(s)

and product(s) which, in turn, would change the rates in the flanking steps, and so on. The rates of production, i.e. the fluxes, in different parts are therefore interdependent. In a system at steady state (which is experimentally and theoretically the most profitable to consider), the change in one enzyme activity (or the comparison of two strains differing at that step) results in a new steady state. This would consist of a re-adjustment of all the fluxes; some, of course, more than others. The net result is therefore a systemic property and not simply that of the enzyme in question and its immediate catalytic consequence.

To test how sensitive the whole system (or any of its associated fluxes) is to changes in one of its enzymes, we can either increase or decrease the enzyme activity and measure the consequences. The comparison of strains with progressively different enzyme activities at one step usually reveals a strongly non-linear relationship between enzyme activity (as measured in cell-free extracts or in permeabilized cells) and systemic flux (as measured on the *in vivo* flow of metabolites) [1,2].

DOWN-MODULATION EXPERIMENTS

This relationship is well illustrated by measurements on the tryptophan synthesis system in *Saccharomyces cerevisiae*, in which five enzymes (see Fig. 2) were down-modulated individually [3–5] (Fig. 1).

The constructs in tetraploid yeast were made to give a series of strains with 4, 3, 2, 1 and 0 doses of a given *TRP* gene. The measured extracted enzyme activities gave values fairly close to those expected from the gene dose. On the other hand, when the effects of such decreases in enzyme activity were measured as in the *in vivo* effect on the growth rate constant (which must be related to the flux to tryptophan), the results showed a non-linear response. The absence of data between about 25% and zero enzyme activity is due to the tetraploid method employed. There is, however, evidence that a leaky mutant, having about 8% anthranilate synthase activity, grows at a decreased rate of 85% of the wild type (Fig. 1a, ■) (P. Niederberger, unpublished work). Both the reduction in the growth rate of this mutant and

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Fig. 1. Down-modulation of enzyme activities in the tryptophan synthesis system

Five separate series of tetraploid strains were constructed, in each of which the four wild-type alleles (+), encoding a particular Trp enzyme, were substituted with non-leaky null mutant alleles (–), giving the genotypes + + + +, + + + –, + + – –, + – – – and – – – – [3]. The different strains were grown in MV-minimal medium [3], and the exponential growth rate μ was determined. From each strain mid-exponential phase cells were permeabilized (for details see [4]) and the down-modulated enzyme activity was determined. Enzymes assayed were: (a) anthranilate synthase (*trp3C*), (b) phosphoribosyltransferase (*trp4*), (c) phosphoribosylanthranilate isomerase (*trp1*), (d) indoleglycerol phosphate synthase (*trp3B*), (e) tryptophan synthase (*trp5A*). Down-modulation of anthranilate synthase was done with a *trp3C* mutant allele (a mutational change in the 5'-half of the *TRP3* gene encoding the Gln-dependent anthranilate synthase subunit prevents the formation of a complex with the *TRP2*-encoded ammonia-dependent anthranilate synthase [5]). An additional point, ■, in (a) is a value from a leaky mutant with 8% synthase activity. Curves were calculated as best-fit hyperbolic functions.

the decrease in the lowest enzyme concentration of the phosphoribosyltransferase (Fig. 1b) are abolished when these strains are grown with tryptophan supplement in the medium. This shows that the decreases in enzyme activity affect the tryptophan flux, which is then reflected in the growth rate. A very similar situation has been reported for argininosuccinase in the arginine pathway in *Neurospora* [6], where a direct measurement of the arginine flux was made. The same non-linear response has also been found with other enzymes of the arginine pathway of *Neurospora* [6–8], as in *S. cerevisiae* and in many other systems [1].

Quantitative analysis

It is evident that the response of the flux to changes in enzyme activity is different at different levels of the enzyme. Furthermore, this response can vary from enzyme to enzyme. To understand these relationships, a radically different approach to that usually taken in traditional biochemistry has been developed [2,9–11]. It required new concepts and new algebraic approaches. The rather dubious concept of the rate-limiting step, key enzyme, pacemaker, etc. has been replaced by the new quantitative concept of the control coefficient. When considering an *in vivo* system where all pathways are embedded in the whole metabolic network, the physiological question which had to be addressed was: what change in the steady-state flux of a particular pathway, δJ , is brought about by a change, δE , in the concentration (or activity) of a given enzyme located anywhere in the system? Clearly, the ratio of these two changes can be regarded as a quantitative measure of the effectiveness of that particular enzyme, E , in controlling the dependent flux J , i.e. $\delta J/\delta E$ is such a measure.

The value of this ratio, however, will depend on the arbitrary units chosen to express both changes and could therefore take any size. If, on the other hand, we use relative changes, normalized

by dividing by the original values, we obtain $(\delta J/J)/(\delta E/E)$. This is a dimensionless number, independent of the choice of units, and hence is much more useful in describing the effect. It also makes it possible to compare the effects of the five different enzymes on the same flux directly.

Another point concerns the size of the change δE . Because of the non-linearities found experimentally (e.g. see Fig. 1) and expected theoretically [1,2], the approach has to consider small changes. The smaller the change imposed, the more accurately will the ratio reflect the response of the flux at the enzyme concentration which exists *in vivo*. In the limit, when we consider infinitesimal changes, we obtain a differential and the ratio becomes:

$$C_{E_i}^J = (\partial J/J)/(\partial E_i/E_i) \quad (1)$$

This ratio of fractional (or percentage) changes is called the flux control coefficient, C , and is in fact the slope of the curve relating % system flux to % enzyme activity. It is clear from the varying slopes of Fig. 1 that the same enzyme can exert a large or a small control on the flux (i.e. have a high or a low value of the control coefficient) depending on its activity, while all other enzyme activities in the sequence remain unchanged.

All the slopes at the wild-type enzyme complement (100% = 4 *TRP* gene doses in the tetraploid) in Fig. 1 are exceedingly small and hence have very small individual control coefficients, with the moderate exception of phosphoribosyltransferase (Fig. 1b). From the data of Fig. 1, we can calculate (or measure) the control coefficients of growth on enzyme activity. These coefficients at wild-type activity of the enzymes are, of course, estimates and are subject to experimental error. They are: $C_a^J = 0.018$; $C_b^J = 0.174$; $C_c^J = 0.013$; $C_d^J = 0.013$; $C_e^J = 0.040$. These very small coefficient values suggest that the tryptophan flux may be equally insensitive, but this remains to be tested. This considerable buffering capacity towards changes in single en-

zymes is not an inevitable situation, and different systems may show a different distribution of the magnitudes of the coefficients [12–14]. Other factors which influence the magnitude of the coefficient are external conditions. Thus, in the often quoted paper of Walsh & Kashland [15], it was shown that variations in citrate synthase activity had a very large effect on Krebs cycle flux when *Escherichia coli* was grown on acetate as the sole carbon source, while the same enzyme variation had virtually no effect in *E. coli* grown on acetate-plus-glucose. This emphasizes the point that the control coefficient is a truly systemic variable.

For any one flux there are, of course, as many control coefficients as there are enzymes. It is not appropriate to discuss here the now very large body of theory which underpins the approach of metabolic control analysis (for reviews see [9,10,16]). One result, however, must be mentioned. It is the well-known flux summation theorem [2,9,11,17] symbolized as follows:

$$\sum_{i=1}^n C_{E_i}^J = 1 \tag{2}$$

The sum of all the coefficients of any one flux is equal to unity. Furthermore, taking a restricted number of enzymes, the effects of the individual small enzyme changes are additive. Thus the sum of the previously estimated individual coefficient is 0.258, and this would be the value found for the group coefficient if all five enzymes were simultaneously changed. In a straight chain of transformations all coefficients are positive, but in a system with a more complex structure, e.g. having branch points, negative coefficients will be present [9]. Nevertheless, relationship (2) (or its more general version) holds for any system of any size or complexity.

The important consequence of the flux summation theorem is that it implies that, for any one flux (or output), there is a distribution of control or sensitivities shared among the enzymes. This means that, in general, there will be a number, and sometimes a large number, of enzymes that contribute to the control of a chosen flux. In such a situation, which may in practice be quite common, no single variation in enzyme activity may alter the magnitude of the flux to any significant extent. This seems to be the case for the tryptophan pathway enzymes in Fig. 1. The remainder of the control coefficients ($1 - 0.258 = 0.742$) will be distributed over the whole metabolic network, beginning with glucose uptake and involving possible negative coefficients (e.g.

enzymes in the other aromatic branches). While the rest of the control coefficients sum to 0.742, we do not know what the distribution of their values is. It could be a ‘flat’ distribution, whereby each individual coefficient would be very small. If, on the other hand, the distribution is ‘skewed’, there may well be one (or two) coefficients with significantly higher values. Only an experimental exploration can decide this.

UP-MODULATION EXPERIMENTS

Having probed the sensitivity of the system to enzyme variations (using down-modulations as a method), can we use these quantitative results to predict the impact of up-modulations on the tryptophan flux, which is the principal aim of these investigations? It is technically more difficult to generate a series of increasing activities of the various enzymes. In the following experiments we have essentially used single very large increases in each of the enzyme activities by the use of multi-copy vectors and have measured the flux directly. The same five *TRP* genes used in the previous experiments were put either individually or in different combinations on a 2 μm-based multi-copy vector. The different plasmids were transferred into yeast strains which carried the corresponding *trp*[−] mutations (Table 1).

The recipient strains were slightly modified to enable the tryptophan flux to be easily determined. All strains carried the *aat2* mutation (see Fig. 2), eliminating the principal tryptophan degradation enzyme [18]. There are therefore only two product fluxes to be measured: (1) the tryptophan accumulation rate and (2) the rate of tryptophan incorporation into protein in the growing culture. The sum of both, taking account of the growth rate, gives the total tryptophan flux (details of the methods are given in Table 2a). In addition, the strains were grown in minimal medium containing 10^{−4} M-anthranilic acid (AA). This was done because anthranilate synthase is feedback-inhibited to 75–80 % even at the very low tryptophan concentrations in wild-type cells [3]. Any further possible increase in tryptophan flux, and hence concentration, thus would have been counteracted by the feedback effect. An up-modulation of the feedback-sensitive *TRP2* gene would create only a minimal increase in endogenous flux from chorismic acid to anthranilic acid, and eventually to Trp [19], and thus was not done. This procedure, of course, adds an additional flux to internal anthranilic acid and effectively

Table 1. Strains and plasmids

All strains carry mutation *aat2* eliminating the principle Trp degradation enzyme [18]. The plasmids are based on the 2 μm vectors pJDB207 and pJDB219 with the *LEU2d* gene as selectable marker [30]. With those plasmids carrying more than two *TRP* genes, the copy number apparently was too low to compensate for the extremely low expression of the promoter-deleted *LEU2d* gene, which made it necessary to add leucine to the minimal MV-medium (see also Tables 2a and 2b).

Strain and plasmid	Genotype of strain	Genotype of plasmid	Reference
RH805	<i>aat2</i>	–	[18]
RH1196 (pME530)	<i>trp4 leu2 aat2</i>	<i>TRP4</i>	[29]
RH1199 (pME526)	<i>trp1 leu2 aat2</i>	<i>TRP1</i>	[19]
RH1194 (pME550)	<i>trp3 leu2 aat2</i>	<i>TRP3</i>	[23]
RH1195 (pYAS-1)	<i>trp5 leu2 aat2</i>	<i>TRP5</i>	A. Strasser, unpublished work
RH1201 (pME529)	<i>trp1 trp3 leu2 aat2</i>	<i>TRP1 TRP3</i>	U. Wirth, unpublished work
RH1203 (pME553)*	<i>trp2 trp1 trp3 leu2 aat2</i>	<i>TRP2 TRP4 TRP1d† TRP3</i>	[23]
RH1203 (pME557)	<i>trp2 trp1 trp3 leu2 aat2</i>	<i>TRP2 TRP1 TRP3</i>	[19]
RH1203 (pME558)*	<i>trp2 trp1 trp3 leu2 aat2</i>	<i>TRP2‡ TRP4 TRP1 TRP3</i>	[19]
RH1207 (pME554)	<i>trp4 trp1 trp3 trp5 leu2 aat2</i>	<i>TRP2 TRP4 TRP1d TRP3 TRP5</i>	[23]
RH1207 (pME559)	<i>trp2 trp4 trp1 trp3 trp5 leu2 aat2</i>	<i>TRP2f TRP4 TRP1 TRP3 TRP5</i>	[19]

* In these two constructs no strain with a *trp4* mutation was available, which adds one chromosomal *TRP4* gene dose to the plasmid-encoded copies.
† *TRP1d* symbolizes the *TRP1* *EcoRI* fragment [31] with a partially deleted promoter, showing only about 10–20 % activity. Contrary to this the *TRP1* gene contains an extended and fully active promoter [19].
‡ *TRP2f* symbolizes a *TRP2* mutant allele encoding a feedback-resistant, but catalytically normally active, anthranilate synthase [3].

Table 2. Trp accumulation and flux upon up-modulation of single (a) and multiple (b) *TRP* genes

Wild-type enzyme levels were as follows (nmol·min⁻¹ mg of protein⁻¹): anthranilate (AA) synthase, 1.5; phosphoribosyl (PR)-transferase, 1.1; phosphoribosyl anthranilate (PRA) isomerase, 5.1; indoleglycerol phosphate InGP synthase, 1.7; tryptophan synthase (A+B activity), 1.4. Activities were determined in Triton X-100-permeabilized cells as described earlier [3]. The Trp accumulation rate (nmol·min⁻¹ mg of protein⁻¹) was determined in cells growing in MV-medium containing Leu and Val (30 µg/ml each) and 10⁻⁴ M-anthranilic acid as described earlier [19]. The accumulation rate of Trp in protein was determined on the basis of a Trp content of yeast protein of 60 nmol/mg [32]. Total flux to Trp was calculated as the sum of the Trp accumulation rate in the free pool and Trp incorporated into protein in proportion to the growth rate. All strains, including the reference wild-type strain RH805, carry mutation *aar2*, eliminating aromatic aminotransferase II, the first step in Trp degradation [23]. The complete genotype of the yeast recipient strains and of the plasmids is indicated in Table 1. No up-modulation experiment with the *TRP2* gene alone was done because this enzymic step was bypassed, mainly by feeding on anthranilic acid.

Plasmid	Up-modulated genes	Relative enzyme levels					Flux to tryptophan			
		AA synthase (<i>TRP2</i>)	PR- trans- ferase (<i>TRP4</i>)	PRA isomerase (<i>TRP1</i>)	InGP synthase (<i>TRP3</i>)	Trp synthase (<i>TRP5</i>)	Trp accu- mulation rate	Trp accu- mulation in protein	Total Trp flux	Total relative flux
(a)										
Wild-type		1.0	1.0	1.0	1.0	1.0	0.10	0.33	0.43	1.0
pME530	<i>TRP4</i>	1.2	19.9	1.2	1.6	1.2	0.27	0.30	0.57	1.3
pME526	<i>TRP1</i>	1.5	1.2	10.6	1.6	0.7	0.20	0.30	0.50	1.2
pME550	<i>TRP3</i>	1.5	0.8	1.8	51.5	1.1	0.13	0.31	0.44	1.0
pYAS-1	<i>TRP5</i>	1.3	0.8	1.7	1.6	37.1	0.23	0.29	0.52	1.2
(b)										
pME529	<i>TRP1 TRP3</i>	1.5	0.6	58.6	56.0	0.5	0.58	0.29	0.87	2.0
pME553	<i>TRP2 TRP4 TRP1d TRP3</i>	25.0	34.0	1.7	47.0	1.0	0.72	0.31	1.03	2.4
pME557	<i>TRP2f TRP1 TRP3</i>	29.4	1.0	30.6	40.9	1.0	0.22	0.31	0.53	1.2
pME558	<i>TRP2f TRP4 TRP1 TRP3</i>	26.5	26.4	30.3	37.4	0.8	0.59	0.32	0.91	2.1
pME554	<i>TRP2 TRP4 TRP1d TRP3 TRP5</i>	20.0	15.2	1.6	20.7	18.1	3.25	0.29	3.54	8.2
pME559	<i>TRP2f TRP4 TRP1 TRP3 TRP5</i>	24.0	19.6	22.1	26.9	23.9	3.51	0.28	3.79	8.8

shortens the pathway. It would also result in a change in the coefficient distribution. The results for single up-modulations of the other four Trp enzymes are shown in Table 2(a).

These large changes in enzyme concentrations must be described as ΔE_i (where Δ stands for a large change, as distinct from the infinitesimal changes of classical control analysis). Similarly, the resulting flux change is symbolized by ΔJ_i . Different algebraic relationships apply to such experiments. To obtain a measure of these effects, we define a deviation index, D , which in each case will take the form $(\Delta J_i/J_i)/(\Delta E_i/E_i) = D_{E_i}^{J_i}$. (A similar type of formulation has been used previously [20], though for rather different kinds of systems, and has been called a 'sensitivity amplification factor'.) As can be seen (Table 2a), the effects on the flux are very small. Increases in single enzyme activities of between 10- and 50-fold resulted in increases in total Trp flux of, at most, 30%.

Did we have any expectations for these results? We have some understanding of the very small response of the growth rate, related to the response of Trp flux, when each of the five enzymes was individually down-modulated (Fig. 1). The exceedingly small slopes imply, of course, that small up-modulations would be equally ineffective. These monotonic response curves, approaching a plateau for each of the enzymes, suggest that even individual changes are unlikely to make a great impact, and Table 2(a) shows that these are indeed the experimental findings. We therefore come to the same conclusion as we did from the down-modulation results, namely that individual enzymes have low control coefficients for growth and relatively small deviation indices for flux.

Multiple up-modulations

We have, however, additional expectations when we consider the effects of large changes. It has been shown [21] that, if a system consisting of many enzymes sustains a flux J , then, if all

the enzyme concentrations are multiplied by a factor r (whatever its magnitude), the resulting flux will be $r \cdot J$. This is equivalent to asserting that $(\Delta J_i/J_i)/(\Delta E_i/E_i)^* = 1$, where $[\Delta E_i/E_i]^*$ stands for the simultaneous and equal elevation of all enzymes by an arbitrary factor r . This 'co-ordinate theorem' is analogous to the summation theorem but the individual indices are, in general, not additive.

This has important consequences for the over-production of particular metabolites. When an additional copy of a gene is stably incorporated into the genome, the increase in gene dose is not infinitesimal but by a factor of 2 and, in almost all cases, so is the increase in the concentration of the dependent enzyme. With plasmids, this factor can be very much larger depending on the nature of the multi-copy vector used. If more than one enzyme activity is up-modulated, the results of such DNA manipulations must therefore be analysed in terms of large-finite-change systems, which have the above property of simultaneous equal changes of all enzymes but whose individual contributions of a smaller group of enzymes are not additive (J. R. Small & H. Kacser, unpublished work). Apart from the quite impractical (and undesirable) proposal to increase every enzyme concentration in the organism by such a factor, is this theorem of any practical value? With the very large number of enzymes, and hence coefficients, the average value of any $C_{E_i}^J$ is exceedingly small even if the number of negative coefficients is taken into account. The distribution of coefficient values, however, is likely to be very skewed, with the vast majority being indistinguishable from zero and a small group predicted whose sum makes up a substantial part of the proportional responses. The same applies to the deviation index, D . This effective reduction of the system to a much smaller number of component parts is therefore part of our expectations. It is this small group which we therefore must modulate simultaneously. This group is most likely to be found to be directly involved in the flux

Fig. 2. Aromatic amino acid biosynthesis, including the tryptophan branch

The five *TRP* genes (*TRP2*, *TRP4*, *TRP1*, *TRP3*, *TRP5*) encode the following enzymes: *TRP2*, anthranilate synthase (AAS); *TRP4*, anthranilate phosphoribosyltransferase (PRT); *TRP1*, phosphoribosylanthranilate isomerase (PRAI); *TRP3*, indoleglycerol phosphate synthase (InGPS); including the Gln-dependent AAS subunit coded by *TRP3C*; *TRP5*, tryptophan synthase (TRPS; A + B reaction). Two aromatic amino transferases, *AAT1* and *AAT2*, perform the first step in Trp degradation to tryptophol. For the up-modulation experiments (but not for those represented by Fig. 1), two important changes to the experimental system were made: (1) a mutant allele *aat2* eliminated the high-affinity Trp-inducible enzyme [18]; (2) anthranilic acid (AA) was added to the medium and is taken up through the cell membrane by an unknown mechanism. Other abbreviations: CA, chorismic acid; PA, prephenic acid; CAMU, chorismic acid mutase.

considered, and in areas of metabolism closely coupled with it.

We now report how the tryptophan flux responded when certain combinations of the various *TRP* genes were placed on the same plasmid, leading to an up-modulation of the corresponding Trp enzymes by similar factors (see Table 2b).

In the first four constructs, different combinations of two to four Trp enzymes were up-modulated simultaneously. An increasing effect on Trp flux was observed, but the 'fold change' (relative flux) was in most cases greater than that expected from the fold changes of the individual enzyme elevations (Table 2a). The greatest discrepancy was seen in the strain carrying pME559, where an increase in all five enzymes resulted in a fold change in the Trp flux of 8.8. On the additivity assumption using individual effects, one would expect a fold change of about 2. This demonstrates experimentally the non-additivity of a limited number of simultaneous changes on the flux.

A second point is the discrepancy of there being 'only' an 8.8-fold change. Although considerable, this is far short of the expectations of the co-ordinate theorem of 24-fold changes in the enzymes. The reason for this discrepancy is most likely that other enzyme steps, which carry some flux control, have remained at unchanged levels.

The most relevant enzymes are those of the shikimate pathway which 'feed' the Trp pathway considered here (see Fig. 2). An up-modulation of the *ARO* genes involved in the shikimate pathway, in the same manner as was done with the *TRP* genes, would most likely have brought about an increase in Trp flux. We have some evidence of the possible effects. This was shown by making use of a *gcd2* mutation, which leads to a constitutive de-repression of genes under the general amino acid control [23,24]. This involves in particular the *ARO* genes of the shikimate pathway, but also the *TRP* genes (except for *TRP1*), which are increased in concert by factors of between 2 and 7 in a *gcd2* mutant ([19,21]; P. Niederberger & H.-P. Schmauder, unpublished work). To demonstrate the contribution of the shikimate pathway only, a *trp4* mutation, blocking the pathway after anthranilate acid, was combined with mutation *gcd2*, allowing for a quantitative measurement of the accumulation rates of anthranilic acid and chorismic acid. Indeed, we observed a 1.7-fold increase in this accumulation rate in a *gcd2/trp4* mutant compared with a *GCD2/trp4* mutant (Table 3). The question of to what degree this flux increase would be transmitted to the

complete system is difficult to answer with a *gcd2* mutation, since (as described above) *TRP* genes are de-repressed simultaneously with *ARO* genes. An up-modulation of only the *ARO* genes by genetic engineering, as was done with the *TRP* genes, would give the definite answer. What the results do show is that there is some control on this part of the system and that the 'tryptophan pathway' extends backwards well beyond chorismic acid.

This leaves two other steps which may contribute to the control of the flux. The first is a possible transport system which takes anthranilic acid into the cells and which is part of the experimental system. [We were restricted to 10^{-4} M-anthranilic acid because at higher values a severe substrate inhibition by this compound on phosphoribosyltransferase takes place (results not shown)]. Even without a specific transport enzyme, this step will have a control coefficient for Trp flux. In the absence of data, we cannot give a quantitative assessment of the likely effect.

There is a second factor which, in certain strains, is likely to have a negative effect on the Trp flux as measured. It is the second aromatic amino transferase encoded by gene *AAT1*. Its activity is much lower than that encoded by *AAT2* (which is inactive in our system), and its affinity for Trp is very low ($K_m = 6$ mM) [18]. It can therefore be ignored in most strains. We found, however, that with plasmid pME559 the internal concentration of Trp increased very rapidly above this level [19], and as a consequence Trp degradation will take place and lead to an underestimate of the total Trp flux. The true fold change is therefore greater than 8.8. This negative effect could be eliminated by the introduction of a mutational decrease in *AAT1* activity.

Finally, the step(s) incorporating tryptophan into protein (which have a positive effect on the flux) must be considered, since Trp is not an end product. It would have been extremely difficult (if at all possible) to increase the rate of Trp incorporation into protein. By inhibitor studies on this process, however, it would be possible to estimate the control coefficient and thus get some idea of the likely (hypothetical) effect of stimulating this step.

The apparent failure to demonstrate the predictions of the co-ordinate theorem (24-fold enzyme elevation producing a 24-fold flux elevation) is no doubt due to the very limited number of enzymes subject to up-modulation. The suggested 'improvements' outlined above are most likely to result in a further rise in tryptophan flux. This must, however, remain speculation. What the use of the five-enzyme up-modulation has demonstrated is

Table 3. Effects of de-repression of *ARO* genes on flux

Abbreviations: AA, anthranilic acid; CA, chorismic acid.

Strain and genotype	Enzyme levels (relative)	Accumulation rates (nmol·min ⁻¹ ·mg of protein ⁻¹)			Relative rates
		CA	AA	Total	
RH21 <i>trp4,GCD2</i>	1	0.27	1.91	2.18	1
RH795 <i>trp4,gcd2</i>	2-7	0.25	3.45	3.70	1.7

that, although individually small, their joint elevations produce more than additive effects. This is clearly seen by comparing the individual effects (total relative flux) shown in Table 2(a) with the strains shown in Table 2(b). Single up-modulation results are therefore an insufficient guide for predicting the effects of simultaneous multiple up-modulations. There is a relationship between the deviation index, D_{E_i} , and the control coefficient, C_{E_i} , but it is a complex one. This positive non-additivity for a limited number of steps is theoretically expected in large-finite-change systems (J. R. Small & H. Kacser, unpublished work), and is an important property when considering genetic manipulation with the aim of increasing output.

CONCLUSIONS

The experimentation and analysis of this model system are of course not complete. There are, however, some important conclusions which this work does demonstrate. The control of a particular flux cannot be thought of as restricted to a part of the whole system which, for convenience, is designated by a name (such as the tryptophan pathway). The idea, frequently expressed in the literature, that you need only begin at the first committed step clearly does not correspond to physiological reality.

Secondly, in many systems, of which this may be one, we do not find any single step for which increases (and sometimes very large increases) in enzyme activity will produce significant increases (let alone proportional changes) in a dependent flux. The hunt for the elusive rate-limiting enzyme in such systems is bound to lead to disappointment. Schaaff *et al.* [26] did experiments not unlike the second part of our study. They investigated the glycolytic pathway in yeast, measuring the rate of ethanol production as well as the growth rate. Eight enzymes, including some often described as being regulatory, were individually increased, using plasmids, by factors of between 4 and 14. No significant increases in either of the measured characters were reported in any of the constructs. The general message of our study can therefore be summed up by saying that successful use of genetic manipulation will, in almost all cases, involve the simultaneous elevation of a group of enzymes.

Up-modulation experiments are expensive and involve by no means trivial technical problems. From the results presented here and the insight gained from a systemic view of metabolism, we can devise a kind of strategy which could be of assistance before engaging in cloning exercises. If the magnitudes of the flux control coefficients are known (and there are well-developed methods for determining them [2,6,10,12,13,27,28]), some steps may well be found that, by having a significantly non-zero coefficient, promise to be an effective point for single up-modulation, while those with very small coefficients will not. There is an even simpler way of eliminating the steps with very small coefficients by using classical genetic methods. From the data in Fig. 1, it is clear that a decrease to 50 % of wild-type enzyme activity has virtually no effect on the measured flux. This

is precisely the situation when a heterozygote or heterokaryon + /null is investigated. For the no-effect result, we would describe the null mutant as recessive. Many null mutants are known (or can easily be obtained) and a finding of recessivity will immediately allow us to conclude that we are dealing with a very small control coefficient at the wild-type value [1]. When such an analysis shows that no single enzyme is likely to give a useful increase, we must turn to multiple-step up-modulations. A method for determining the group, or overall, coefficients of a number of steps has recently been developed [27]. These group coefficients may be useful in this respect. In the end, however, we must be guided by experiments which probe particular sections of the system, and here we must be aware that the effective summation of control can extend into parts of metabolism traditionally not considered relevant. Furthermore, the positive non-additivity of the effects of up-modulation of a number of enzymes could make us feel confident that there will be a group of enzymes, not necessarily in contiguous sequence, which will give useful enhancement of a chosen flux.

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